# The roles of CaMKII and Ras-GRF2 in the establishment of drug addiction

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# The roles of αCaMKII and Ras-GRF2 in the establishment of drug addiction

PhD Thesis by Alanna C. Easton

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*I would like to dedicate my PhD Thesis to my*
*Mum and Dad*

*So much time and money spent on my education,*
*and all you got was this lousy book…*
*:)*

*Thank you for all your support,*
*I love you both very much!*

*Alanna*
*xxx*

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**Chapter 2:** A.C.E., C.F., and C.P.M. conceived and designed the study. A.C.E. performed all experiments. A.C.E. performed statistical analysis.

**Chapter 3:** C.P.M. and A.C.E. conceived and designed the study. *Alcohol drinking:* Experiments were performed by A.C.E. and C.P.M., Data was analysed by C.P.M. *Microdialysis:* All experiments were performed and analysed by A.C.E. *Conditioned Place Preference*: All experiments were performed and analysed by A.C.E. *LORR*: All experiments were performed and analysed by A.C.E. *Blood alcohol levels:* Sample collection was performed by A.C.E. Samples were sent for analysis to C.P.M., P.L. and J.K. in Germany. *c-Fos expression*: Sample collection was performed by A.C.E. Samples were sent for analysis to C.P.M., J.S., Y.G., R.R.D. and G.H.M. in Germany.

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## Abstract:

Addiction is a chronic, relapsing disorder associated with health and social problems for the individual and great economic costs for society. Despite significant progress in the understanding of drug effects on the nervous system, major processes involved in the development of addiction are still insufficiently understood. At a cellular level, many drugs of abuse induce a long term potentiation-like state in the dopamine (DA) neurons of the ventral tegmental area (VTA), a process which enhances synaptic transmission and potentiates signals for longer within the cell. This is a major cellular mechanism underlying normal learning and memory formation, but is also important in the formation of drug-related memories and addiction. By adopting a correlative approach to further dissect which neuronal mechanisms contribute to addiction, the focus of this thesis is placed on two separate genes, each of which code for different proteins involved in synaptic plasticity. The thesis aims to assess whether these genes contribute to the development of drug preference and addiction, and the speed at which these behaviours are established. Alpha calcium/calmodulin dependent protein kinase II (αCaMKII) and Ras-specific guanine nucleotide releasing factor 2 (Ras-GRF2) are calcium/calmodulin dependent kinases which play an important role in the plasticity of the glutamatergic and monoaminergic systems by influencing long-term potentiation (LTP).

Transgenic mice were tested through an extensive battery of behavioural (spontaneous behaviours, consumption and conditioned place preference) and in vivo neurochemical (in vivo microdialysis and HPLC-ED) techniques, in order to assess whether αCaMKII and Ras-GRF2 have the potential to control the rate at which addiction related behaviours are acquired and/or established. The current thesis implicates αCaMKII autophosphorylation in the mediation of threat induced activity in response to novel situations and stimuli. αCaMKII also appears to play an important role in the establishment of drug addiction by altering the differential reinforcing effects of alcohol. A deficit in αCaMKII autophosphorylation delayed the rate of alcohol drinking establishment. However, repeated periods of withdrawal and reinstatement allowed for these drinking behaviours to be established to

the same capacity as observed in wild-type mice. Alcohol administration also altered dopamine and serotonin levels in the mesocorticolimbic system of αCaMKII autophosphorylation deficient mice. Adaptations in this system were also found following the development of cocaine preference. Ras-GRF2 has an important role at the synapse and Ras-GRF2 deletion appears to result in the failure of the monoaminergic system to adapt in a typical way in response to alcohol exposure, which may make the animal less vulnerable to the rewarding properties of alcohol. One hypothesis is that the Ras-GRF2 deletion has the potential to serve as neuro-protective factor against alcoholism. Characterisation of the roles of αCaMKII and Ras-GRF2 suggests that the proteins encoded by these genes may contribute to aspects of drug addiction related behaviors by modulating monoaminergic drug responses in the brain.

# Table of contents

# General Introduction

Chapter 1

# 1. Addiction and substance abuse disorders

Addiction is a chronic, relapsing disorder and can be defined as the compulsive use of drugs, despite negative consequences (Hyman, 2005), progressing from impulsive to compulsive use in stages (Koob and Le Moal, 2005). Initially, during the intoxication phase drug taking behaviour is positively reinforced. As consumption increases, this behaviour progresses towards compulsion, any negatively reinforcing effects are offset by drug use, and the alleviation of a negative emotional state increases the probability of response (Koob and Le Moal, 1997). Substance abuse disorders are classified as serious psychiatric disorders by both the Diagnostic and Statistical Manual of Mental Disorders IV (DSM IV), and the International Classification of Diseases (ICD-10). Drug dependence is a debilitating disorder and is associated with huge health and social concerns for the individual and great economic costs for society.

## 1.1. Alcohol abuse

The consumption of fermented beverages, containing alcohol (ethanol), is thought to have existed as early as 10,000 BC. Today, alcohol is consumed recreationally and legally in western societies, despite its potential for harmful use and abuse. Alcohol is consumed for a variety of reasons, although it has been suggested that the key component to compulsive drug seeking is the alleviation of a negative affective state (Koob, 2011), other factors such as the inflation of “self-perceived survival ability and reproductive fitness” (Newlin, 2002) are likely to play a role in the decision to consume alcohol. Of course, not everyone consuming alcohol will become addicted (Zinberg and Jacobson, 1976; Zinberg *et al.*, 1978; Glynn *et al.*, 1983; O'Malley and Johnston, 2002). The concept of ‘drug instrumentalization’ (Müller and Schumann, 2011), suggests that psycho-active drugs are consumed for their desirable effects on mental states, for example, in order to enhance everyday functioning by influencing mood, by reducing stress, and increasing sociability (Chick, 1999; Peele and Brodsky, 2000; Molnar *et al.*, 2009). Approximately 55% of the global adult population legally consume alcohol. The World Health

Organisation (WHO) estimate the number of people who currently use alcohol worldwide to be 2 billion, of these people 76.3 million have an alcohol use disorder (WHO, 2004). In approximately 10-20% of alcohol consumers, chronic use and abuse will lead to serious health problems such as damage to organs such as the liver, pancreas and the brain (Spanagel, 2009). The Global Information System on Alcohol and Health (GISAH, 2011) cite alcohol as having a significant causal role in 60 different types of disease, and can account for 9% of deaths in the 15-29 age group (WHO, 2011). Despite alcohol being a legally consumed drug of abuse, harmful use results in 2.5 million deaths each year (WHO, 2011). In addition, it has been shown that approximately 10% of an industrialized nation's gross domestic product is spent in connection with alcohol use and abuse (Nutt *et al.*, 2007), and each year Europeans alone spend approximately 100 billion Euros on alcoholic beverages (Spanagel, 2009).

There are many targets for ethanol within the brain (McBride *et al.*, 2002; Harris *et al.*, 2008; Spanagel, 2009). One way in which alcohol is known to exert its effects is mediation via the glutamatergic system, in particular the N-methyl-D-aspartate (NMDA) receptors (Spanagel, 2009). Current flowing through the NMDA receptors is known to be reduced during acute ethanol exposure (Lovinger, 1997; Narahashi, 2000). NMDA receptors are a major constituent of the post-synaptic density, and are highly expressed in neurons (Colbran and Brown, 2004; Irvine *et al.*, 2006; Wayman *et al.*, 2008). Calcium ($Ca^{2+}$) entry into the post-synaptic cell, through NMDA receptors, results in activation of a number of downstream molecules, such as calcium calmodulin dependent protein kinase II (CaMKII), a protein kinase required for induction of long term potentiation (LTP). NR1 and NR2 NMDA subunits have been identified as the main regulators of channel sensitivity to ethanol (Ronald *et al.*, 2001; Ren *et al.*, 2003). Interestingly, the NR2B subunit of the NMDA receptor has both binding and phosphorylation sites for CaMKII, indicating that NMDA receptor sensitivity may be affected by an interaction with CaMKII (Xu *et al.*, 2008). Alterations in channel sensitivity to ethanol have been shown to vary across brain regions (Simson *et al.*, 1991; Yang *et al.*, 1996).

Importantly, ethanol acts as an NMDA antagonist in certain brain regions including the ventral tegmental area (VTA) and the substantia nigra, both dopamine (DA) rich areas of the midbrain which have implications for motivation, reward and addiction (Yang *et al.*, 1996).

There are large numbers of transmitter systems and synaptic mechanisms involved in the establishment of alcohol dependence (Nestler and Aghajanian, 1997; Heilig and Koob, 2007). The γ-aminobutyric acid (GABA) system is the major inhibitory transmitter system in the brain and it has been suggested to mediate the rewarding and anxiolytic effects associated with ethanol consumption (Koob *et al.*, 1998a; Stephens *et al.*, 2005). It has been postulated that changes in this circuitry may provide motivation for excessive drinking, and the transition from casual alcohol use to dependence (Koob *et al.*, 1998a). It is $GABA_A$ receptor function that is enhanced by ethanol, the extent of which strongly depends on the subunit composition of the $GABA_A$ receptor (Stephens *et al.*, 2005; Spanagel, 2009). Evidence suggests it is the α and β subunits in particular which are involved in the actions of ethanol (Mihic *et al.*, 1997). Lending support to the theory that GABA activation is an important component of the acute reinforcing effects of alcohol, the acute blockade of $GABA_A$ receptor function can inhibit the motivation for responding for ethanol (Koob, 2004). Acutely, alcohol administration is known to enhance DA release from the mesolimbic pathway (Di Chiara and Imperato, 1988) with preferential release from the nucleus accumbens (NAcc) (Pontieri *et al.*, 1995). It has been postulated that this occurs via GABAergic feedback into the VTA, decreasing the activity of the GABAergic neurons, leading to a disinhibition of mesolimbic DA neurons (Spanagel and Weiss, 1999). $GABA_A$ receptor inhibition is mediated by several protein kinases (Moss and Smart, 1996; Brandon *et al.*, 2002; Kittler and Moss, 2003), one of which is CaMKII. CaMKII can bind to, and phosphorylate, different isoforms of $GABA_A$ receptors, resulting in alterations in $GABA_A$ receptor expression and function (Houston *et al.*, 2009). CaMKII is capable of either prolonging or increasing the amplitude of inhibitory post-synaptic currents (IPSC) at $GABA_A$ β2 subunit-containing receptors, and has been implicated in the trafficking of $GABA_A$ receptors (Houston *et al.*, 2009).

CaMKII is known to play an important role at excitatory synapses (Sheng and Hoogenraad, 2007), associating with a number of different proteins and potentially altering synaptic activity (Strack *et al.*, 2000; Robison *et al.*, 2005) and neuronal excitability, determining the extent of inhibitory synaptic plasticity.

### 1.2. Cocaine abuse

Peruvian Indians originally believed that the coca plant was a gift sent from the gods. Cocaine, in its current form, has only been in use for the past 100 years, but the leaves of the coca plant have been chewed by people for thousands of years. Cocaine is the main alkaloid of *Erythroxylon coca*, a shrub which grows abundantly in parts South America, but the main constituent wasn't isolated until the 1850's (Johanson and Fischman, 1989). Sigmund Freud believed cocaine was a cure for a variety of physical and psychological illnesses and it was used in medicine as a local anaesthetic and vasoconstrictor (Barash, 1977). The consequences of chronic use and the potential for abuse were soon discovered and use was limited. Cocaine has also been used in consumable products in the past. The Coca-Cola Company launched 'Coca-Cola' in 1886 and used coca leaves, left over from the cocaine extraction process, as part of their recipe leaving only trace amounts of cocaine present in the beverage**.** In 1903 cocaine was removed from the recipe. Cocaine was used prolifically up until the early 1900's when laws were introduced to control its use. The drug was banned in 1914, resulting in a decrease in consumption up until the 1960's. Consumption increased again during the 70's and 80's due to the popularisation of crack cocaine or freebase and has since become a substantial public health problem, resulting in a large number of medical, psychological and social problems. The United Nations office on Drugs and Crime (UNODC) have estimated that the prevalence of cocaine use worldwide in 2009 ranged from 0.3% to 0.5% of the population aged 15-64, approximately 14.2 to 20.5 million people (UNODC, 2011). Today, cocaine is the most widely used illicit psycho-stimulant drug in the UK, with a prevalence rate of lifetime use of between 3% and 5%

(UNODC, 2011). However, the largest cocaine market continues to be the United States, accounting for 36% of global consumption (UNODC, 2011).

Cocaine is a psycho-active drug and is classified as a stimulant since it enhances activity in the central nervous system (CNS). Cocaine suppresses the appetite and increases locomotion and feelings of alertness, pleasure, well being and euphoria. Cocaine can also induce feelings of paranoia, anxiety and restlessness. Cocaine's effects are experienced almost immediately after administration; this 'high' is short lived and typically lasts for between 30 and 60 minutes.

Cocaine's main system effects are on the monoaminergic system. Cocaine artificially increases extracellular DA levels by binding to the monoamine transporters located pre-synaptically, thus prevents the binding of the monoamines themselves and blocking the re-uptake of DA, serotonin (5-HT) and noradrenaline (NA) (Uhl *et al.*, 2002; Hall *et al.*, 2004; Jones *et al.*, 2009). This causes the extracellular space to become flooded with the transmitter molecules (Pifl *et al.*, 1995; White and Kalivas, 1998) and the subsequent over stimulation of the receptors (Nestler, 2005). Cocaine has a similar affinity for DA, 5-HT and NA transporters (Chen *et al.*, 2006; Han and Gu, 2006) although the most extensively studied mechanism has been the dopaminergic transmitter system (Sofuoglu and Sewell, 2009), and is thought to be the most clinically relevant (Nestler, 2005). There is a high concentration of DA neurons in the limbic system, and this is the site where cocaine predominantly produces its psychoactive effects (Koob *et al.*, 1998a; Hyman and Malenka, 2001; Nestler, 2001; Kalivas and McFarland, 2003). DA D1 (DRD1) and DA D2 (DRD2) -like receptors are primary targets for altering neurotransmission when challenged with cocaine (Montague *et al.*, 2004; Hyman *et al.*, 2006).

The glutamatergic system has also been identified as a cocaine target (Uys and LaLumiere, 2008) and a substantial amount of research is now emerging, looking at the role of the glutamatergic system in the mediation of addictive behaviours (Kelley *et al.*, 2003; Kauer and Malenka, 2007; Gass and Olive, 2008; Uys and LaLumiere, 2008). Cocaine does not act directly on

glutamatergic neurons in the same way it does with DA, 5-HT and NA (Ritz *et al.*, 1990). Literature indicates that cocaine indirectly influences glutamate transmission in the limbic system, including the NAcc, and produces persistent changes in neuronal function which alter the behavioural effects of cocaine (Gass and Olive, 2008; Thomas *et al.*, 2008; Uys and LaLumiere, 2008). While an acute cocaine injection does not seem to have a dramatic effect on glutamate levels in the NAcc (Pierce *et al.*, 1996), cocaine experienced rats increase glutamate levels in the NAcc core in response to cocaine administration (Pierce *et al.*, 1996; Cornish and Kalivas, 2000; McFarland *et al.*, 2003). Cocaine withdrawal after chronic use has been shown to deplete basal levels of glutamate in the NAcc (Pierce *et al.*, 1996; Baker *et al.*, 2002). Taken together, this data suggests that cocaine use influences neuro-adaptations in glutamatergic signalling in the NAcc by acting on monoaminergic transmitters (Schmidt and Pierce, 2010). Both NMDA (Lu *et al.*, 2003; Kelley, 2004; Famous *et al.*, 2007) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) (Cornish and Kalivas, 2000; Self and Choi, 2004; Suto *et al.*, 2004; Schmidt and Pierce, 2010) glutamate receptors contribute to the plasticity at glutamatergic synapses and the reinforcing effects of cocaine. NMDA receptor inhibition prevents LTP in several brain regions (Malenka and Bear, 2004) and at the same time prevents conditioned place preference (CPP), self-administration and behavioural sensitisation to drugs of abuse (Kalivas and Alesdatter, 1993; Schenk *et al.*, 1993; Harris and Aston-Jones, 2003). After glutamate binds and removes the voltage-dependent magnesium ($Mg^{2+}$) block, the NMDA receptors become active and allow $Ca^{2+}$ ions to flow into the post-synaptic cell. This triggers an intracellular signalling cascade which includes a variety of protein kinases, most notably CaMKII (Malenka and Nicoll, 1999). Data strongly implicates the monoaminergic and glutamatergic systems, in conjunction with learning and memory processes, in the development of cocaine related addictive behaviours.

## 2. Are our genes to blame?

Addiction is seen to run in families (Cotton, 1979; Guze *et al.*, 1986) and the individual risk of developing alcoholism increases with the proximity and number of affected individuals (Dawson *et al.*, 1992). The way in which addictions are inherited shows no obvious pattern of Mendelian transmission (Merikangas and Avenevoli, 2000) but studies on large samples of twins can help to tease apart genetic and environmental influences on addictive processes (Kendler *et al.*, 2003). Both environmental and genetic factors play a role in the development of addictive disorders, and contribute to individual differences in vulnerability to the progression from drug use to dependence (Goldman *et al.*, 2005; Ripley and Stephens, 2011). Twin studies help to assess the relative importance of genetic, shared and non-shared environmental influences on complex traits and behaviours. The classical twin design compares the similarity of monozygotic (identical) and dizygotic (fraternal/non-identical) twins. Monozygotic twins are genetically identical and have a high degree of environmental similarity. Dizygotic twins also share their environments but only share 50% of their genes. Large scale twin studies allow the contribution of genes, shared and unique environments to be disentangled. Therefore, if a condition was to be considered heritable it would be expected that more co-twins would be affected in monozygotic twin pairs when compared with dizygotic twin pairs. This has been reported in the majority of twin studies that have investigated the heritability of addictive behaviours (Ball and Collier, 2002; Ball *et al.*, 2007). Genetic studies provide important information regarding hereditary influences and the risk of addiction (Ball, 2008). Addictions are among the most heritable of complex psychiatric disorders, with heritability estimates ranging from 0.39 (39%) for hallucinogens to 0.72 (72%) for cocaine (see Figure 1.1; taken from (Goldman *et al.*, 2005).

Meta-analysis of genetic association studies report specific genetic loci and variants which have been associated with drug addiction. However, it can be difficult to interpret the results since studies can be inconsistent, sometimes analytically biased and under-powered (Li *et al.*, 2011). Meta-analyses of drug

addiction studies have been rather limited and the majority of studies have focused on candidate genes instead of genome wide analysis. While data from human studies can be conflicting, and epidemiological factors can make it hard to demonstrate causal relationships, the use of transgenic and knock-out (KO) animal models has made the investigation of the potentially genetic risk factors for addiction easier. Despite this, there are still a large number of genes/genetic factors currently being investigated in relation to the development of addictive behaviours, several of which mediate normal functioning of the mesocorticolimbic system. The metabolising enzyme monoamine oxidase A (MAOA) is responsible for breaking down DA, 5-HT and NA (Goldman *et al.*, 2005). 5-HT is implicated in impulsivity, anxiety and alcoholism. MAOA is responsible for the breakdown of 5-HT and recent studies have shown that maltreatment and adversity experienced by young boys, combined with low MAOA gene expression levels, can predict conduct problems and lead to addiction (Caspi *et al.*, 2002; Foley *et al.*, 2004). This suggests that low MAOA expression levels might confer some increased susceptibility to alcoholism in certain individuals. Catechol-O-methyl transferase (COMT) also has a major role in the metabolism of DA and NA in the CNS (Enoch, 2006), and the Val158Met polymorphism of COMT results in variation in enzyme activity. The COMT alleles have been implicated in alcoholism, but this effect appears to be population dependent. For example, the Met158 allele is associated with increased drinking in European Caucasian men (Tiihonen *et al.*, 1999; Hallikainen *et al.*, 2000). However, the same allele confers a protective effect against alcoholism in Plains American Indians (Enoch *et al.*, 2006). The Met158 allele may therefore be considered a vulnerability factor for alcohol drinking in certain societies. As mentioned earlier in this chapter, both NMDA (Lu *et al.*, 2003; Kelley, 2004) and AMPA (Cornish and Kalivas, 2000; Self and Choi, 2004) glutamatergic receptors contribute to the plasticity at glutamatergic synapses and the reinforcing effects of cocaine and other commonly abused drugs. A large number of drugs of abuse are known to induce LTP in DA neurons of the VTA (Saal *et al.*, 2003; Thomas and Malenka, 2003; Kauer, 2004) and these changes are mediated, at least in part, by AMPA and NMDA receptors. For example,

blockade of NMDA receptors in the prefrontal cortex (PFC) inhibits extinction learning of amphetamine CPP (Hsu and Packard, 2008), and AMPA infusion into the infralimbic cortex blocks cocaine reinstatement of cocaine seeking behaviour (Peters *et al.*, 2008). Also acting in the frontal regions of the brain are several genetic factors thought to mediate addictive processes, one of which is ΔFosB, a transcription factor which accumulates in the NAcc following exposure to natural rewards such as sucrose drinking. Levels also increase in response to chronic exposure to all drugs of abuse and is thought to contribute to a state of sensitisation (Nestler *et al.*, 2001; McClung *et al.*, 2004; Hyman, 2005). Over expression of ΔFosB in the NAcc increases behavioural responses to cocaine, opiates and other natural rewards such as sucrose. Accordingly, blockade of ΔFosB function in the NAcc suppresses behavioural responses to cocaine (Nestler *et al.*, 2001; McClung and Nestler, 2003; McClung *et al.*, 2004). Brain-derived neurotrophic factor (BDNF) plays a role in the growth and differentiation of developing neurons (Chao, 2003; Chao *et al.*, 2006) and is implicated in learning and memory and synaptic plasticity (Chiocco *et al.*, 2007; Liu *et al.*, 2008). BDNF can contribute to drug taking behaviours including; LTP and long term depression (LTD) in the VTA (Thomas *et al.*, 2001; Mameli *et al.*, 2009), drug induced structural plasticity in mesocorticolimbic DA areas (Robinson and Kolb, 2004; Russo *et al.*, 2009) and drug induced MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinases) signalling in the NAcc and amygdala (Lu *et al.*, 2006; Girault *et al.*, 2007). Alcohol metabolising genes also have the potential to indirectly influence vulnerability to substance abuse disorders. Variations in these genes may contribute to the development of alcoholism. Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are directly involved in the breakdown of alcohol and decreased efficiency of these proteins can cause the build up of alcohol's metabolic products, causing a facial flushing reaction. This is predominantly seen in the Southeast Asian population, discouraging alcohol intake and thereby conveying an indirect protective effect against alcoholism (Thomasson *et al.*, 1991; Radel and Goldman, 2001).

Although genetic factors clearly play a role in mediating addiction, the environment also plays a substantial role in the development of the complex trait of addiction. Further investigation is required into the underlying causes of alcohol and cocaine use, abuse and dependence, and to what degree an individual's genetic make-up will play a role in the development of the disorder.

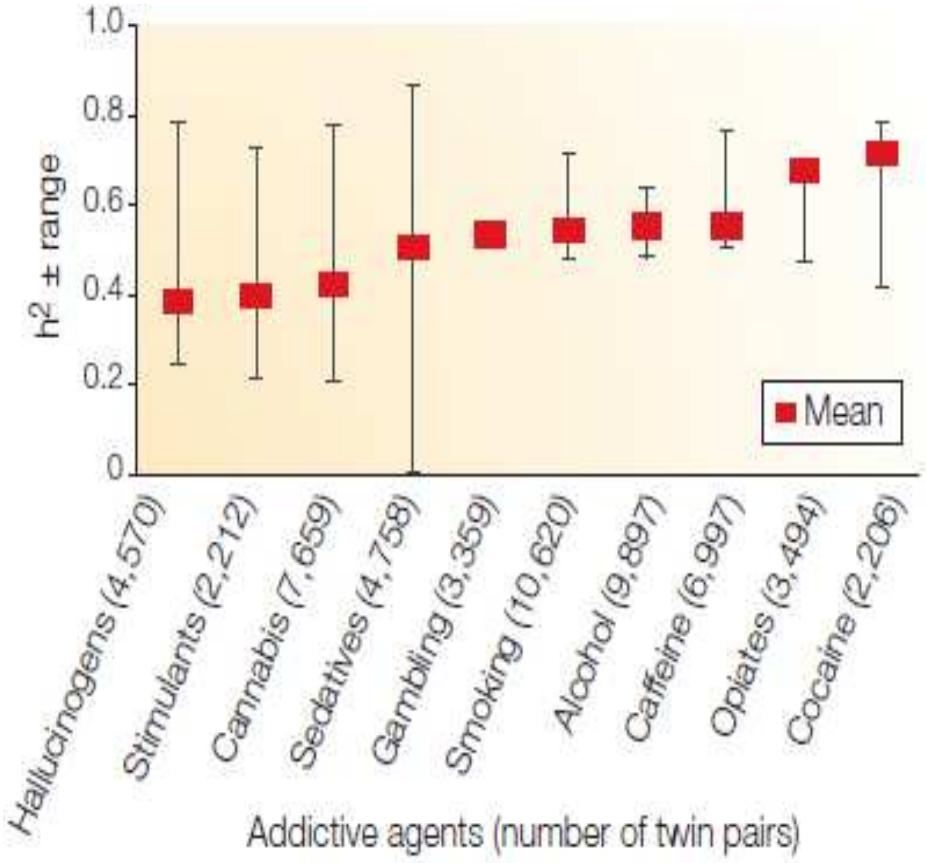


**Figure 1.1:** The heritability ($h^2$) of ten addictive disorders in a cohort of monozygotic and dizygotic twins. An $h^2$ value of 0.4 suggests that genetics make a 40% contribution to the development of the disorder in question. Cocaine and opiates are among the most heritable addictive substances. Taken from (Goldman *et al.*, 2005).

## 3. The learning of addictive behaviours

The process by which addiction is established remains largely unknown, however it is thought to involve a number of learning and memory processes. Wikler and Pescor (1967) have cited “pathological learning” as a model for addiction, although the idea of addiction as a memory was first introduced in 1972 (Mello, 1972). Since then, numerous researchers have attempted to summarise and characterise how the reinforcing effects of drugs may interfere with learning and memory pathways. White (1996) proposed three reinforcing actions, which can be influenced by psychoactive drugs, affecting learning and memory systems: conditioned incentive learning; declarative learning; habit/stimulus-response learning. ‘Conditioned incentive learning’ was first described by Pavlov (1927) and involves the presentation of a neutral stimulus with a reinforcer which elicits an internal state perceived as rewarding, in time the originally neutral stimulus can evoke the same response in the absence of the reinforcer. The CPP test design is commonly used in the laboratory today and is based upon the theory of ‘conditioned incentive learning’, in which a neutral environmental cue becomes associated with a motivationally significant event such as drug experience (Cunningham *et al.*, 2006), thereby eliciting a motivational response. This response is then used to assess the significance/preference of the motivationally significant event. The second reinforcing action described by White (1996) is ‘declarative learning’. The concept was originally described by Tolman (1948; 1949) and was known as ‘stimulus-stimulus’ since it involves learning about relationships among cues and events and is used to illicit appropriate behaviours when required. The neural basis of learning suggests that the hippocampal system can acquire information about the relationship between external cues and internal states (Hirsh *et al.*, 1978; Hirsh *et al.*, 1979; Davidson and Jarrard, 1993) and rapidly store the information relevant to the situation in which a drug is administered (for example, in a social setting) and can therefore contribute to addiction. The final memory type described by White (1996), and thought to contribute to the development of addiction is ‘habit/stimulus-response learning’. Stimulus-response associations are the “bonds” between the neural representation of a stimulus and a response. These associations are strengthened slightly each

time the response occurs, resulting in reinforcement and thus the acquisition of 'habit' strength occurs at a relatively slow pace. In order for these associations to affect future behaviour, there must be some process which is acting to give them a degree of permanency. This process is thought to be mediated by the caudate putamen (CPu) (Divac, 1968; McDonald and White, 1993). More recently the classification of 'addiction memories' has been revised, and the different memory types suggested in relation to drug consumption include: a memory of drug effect, a memory of drug use and a memory of addiction (Heyne *et al.*, 2000; Boening, 2001), although it has been suggested that the involvement of Pavlovian incentive learning mechanisms and reward are the forces driving consumption (Orford, 2001). In general, concepts of drug addiction memory focus on the state of addiction, and tend to overlook the immediately preceding processes occurring in the non-addicted individual, which need to happen before addiction is established. This period is critical for the formation of drug-related memories, retrieval of these memories and ongoing drug consumption (Müller and Schumann, 2011).

It is widely believed that addiction and memory formation share a number of molecular and anatomical pathways (Nestler, 2002a; Kelley, 2004; Hyman, 2005; Nestler, 2005). There are several areas of the brain which are affected by drugs of abuse including the VTA, the NAcc, the medial PFC, the ventral pallidum, the dorsal hippocampus and the central gray and lateral amygdala (McBride *et al.*, 1999). These regions are responsible for mediating the rewarding potential of drugs (Koob *et al.*, 1998b; Wise, 1998; Swanson, 2000; Nestler, 2001; Everitt and Wolf, 2002). Drugs of abuse 'hijack' the natural reward system in the brain (Nestler, 2002b; Kelley, 2004; Hyman, 2005; Novak *et al.*, 2010) which has evolved to evaluate the positive and negative reinforcing properties for natural rewards, such as; food, sex, social interaction, danger, fighting for territory and incurring injury, for the purpose of enhancing survival. In each case, where there has been an adaptation of behaviour, there has also been a learning process (Kelley, 2004).

At a cellular level, many drugs of abuse induce a LTP-like state in the DA neurons of the VTA (Bonci *et al.*, 2003; Saal *et al.*, 2003; Thomas and Malenka, 2003; Borgland *et al.*, 2004; Kauer, 2004), a process which enhances synaptic transmission and potentiates signals for longer within the cell. This is a major cellular mechanism underlying normal learning and memory formation, but is also important for drug memories and addiction as well. The present study aims to evaluate whether disruption in two genes, αCaMKII and Ras-GRF2, can alter measures of drug preference, and the rate at which these preferences are established. Specifically this will be carried out by using an *in vivo* approach to look at the effect of disruption of specific proteins which play key roles in synaptic plasticity on the development of preference for alcohol and cocaine

Although involvement of these genes in addition-related behaviours could be explained by their role in the learning and memory circuits, this thesis does not explicitly test this hypothesis.

## 4. Novel targets in the study of addiction

There is an established link between addictive processes and learning and memory pathways that warrants further investigation. Interruption of this circuitry can influence measures of drug preference, and the rate at which addiction is established (Hyman and Malenka, 2001; Hyman, 2005). Arguably one of the most noteworthy mechanisms worth following-up is the alpha $Ca^{2+}$/calmodulin dependent protein kinase II (αCaMKII). Phosphorylation of CaMKII is important for drug addiction (Anderson *et al*., 2008), but the specific involvement of the autophosphorylation mechanism is not yet known. Further investigation may provide evidence for a contribution of the autophosphorylation mechanism to the development of addiction. Also of considerable interest is the $Ca^{2+}$/calmodulin (CaM) binding exchange factor, Ras-specific guanine nucleotide releasing factor 2 (Ras-GRF2). There is evidence to suggest that Ras-GRF2 contributes to learning and memory formation by influencing the induction of LTP (Thomas and Huganir, 2004; Tian *et al.*, 2004; Li *et al.*, 2006; Tian and Feig, 2006). However, Ras-GRF2 has only recently been associated with drugs of abuse (Stacey *et al*., 2012). Ras-GRF2 is a highly speculative candidate for addictions studies, but further studies may provide evidence for a link between this gene and addiction.

### 4.1. alpha $Ca^{2+}$/Calmodulin Dependent Protein Kinase II (αCaMKII)

CaMKII is a major post-synaptic density protein. It plays a key role in synaptic plasticity in the glutamatergic system and is known to be important for learning and memory formation (Fukunaga *et al.*, 1993; Colbran and Brown, 2004; Lengyel *et al.*, 2004). There are two main isoforms expressed in the brain, αCaMKII and beta CaMKII (βCaMKII) (Colbran and Brown, 2004). The expression levels of CaMKII's α and β isoforms are differentially regulated in cultured hippocampal neurons and high levels of synaptic activity have been shown to up-regulate the expression of αCaMKII and down-regulate βCaMKII expression (Thiagarajan *et al.*, 2002). The αCaMKII isoform is most prominently expressed in the forebrain at glutamatergic post-synapses (Lisman *et al.*, 2002; Colbran and Brown, 2004; Irvine *et al.*, 2006; Wayman *et*

*al.*, 2008). αCaMKII is part of the $Ca^{2+}$ signalling cascade in cells and is typically inactive under basal cellular conditions. αCaMKII is made up of 3 domains, a self-association domain, an autoinhibitory domain and a catalytic domain. In its inactive state, the enzyme is locked shut by binding of the autoinhibitory to the catalytic domain (See Figure 1.2, (Irvine *et al.*, 2006). When $Ca^{2+}$ ions enter the post-synaptic cell they bind calmodulin, forming the CaM complex. The CaM complex then activates αCaMKII by displacing the autoinhibitory domain from the catalytic site. Once rendered active, the catalytic site of αCaMKII is free to phosphorylate downstream substrates involved in intracellular signal transduction, but also has the potential to switch to an autonomous or independent mode of activity known as autophosphorylation (Giese *et al.*, 1998). Upon autophosphorylation, αCaMKII is able to phosphorylate neighbouring subunits in the holoenzyme at the threonine 286 residue, even after the dissociation of the CaM complex, allowing the kinase to remain active for longer. This autophosphorylation mechanism prevents αCaMKII from reverting to its resting state even after $Ca^{2+}$ levels drop to baseline levels, the signal ceases and the cell rests. As illustrated in Fig 1.2, during $Ca^{2+}$ independent activation only one CaM molecule is required to activate the entire holoenzyme as opposed to during $Ca^{2+}$ dependent activity when six CaM molecules would be needed to achieve the same result. It is this switch to a $Ca^{2+}$ independent state which allows signals to be potentiated for longer within the cell, leaving a lasting imprint along the pathway, and thereby accelerating learning (Lucchesi *et al.*, 2011).

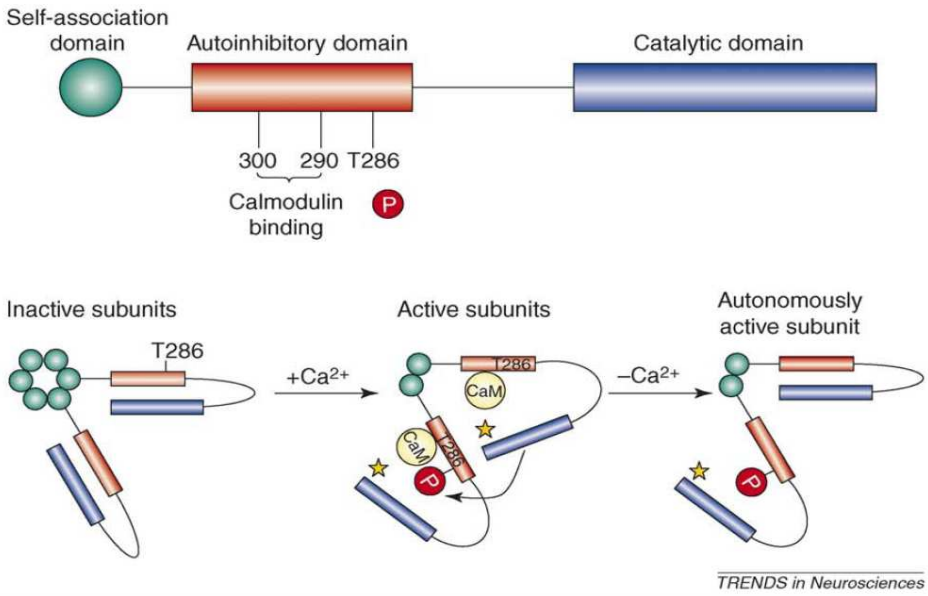


**Figure 1.2:** The αCaMKII autophosphorylation mechanism. Taken from Irvine *et al.*, 2006.

CaMKII is capable of phosphorylating itself and a number of intracellular targets including AMPA receptors (Poncer et al., 2002), NMDA receptors (Bayer et al., 2001), L-type $Ca^{2+}$ channels (Dzhura *et al.*, 2000) and tyrosine hydroxylase (TH ; Griffith and Schulman, 1988). The potential interaction between CaMKII and TH is of particular interest as TH is the rate limiting enzyme in DA synthesis, which in turn is a precursor for NA. TH, CaM and CaMKII are co-localised in the NAcc. It has been suggested that TH activity and DA synthesis are regulated by $Ca^{2+}$/CaM/CaMKII in the NAcc and other areas (Sutoo *et al.*, 2002). There is also a strong link between CaMKII and serotonergic function in the brain. Tryptophan hydroxylase (TPH) is the initial and rate limiting enzyme in the biosynthesis of 5-HT (Kuhn *et al.*, 2007), and it exists in two isoforms, TPH1 and TPH2 (Kuhn *et al.*, 2007). TPH2 is exclusively expressed in the CNS and is activated by CaMKII phosphorylation (Hamon *et al.*, 1977; 1978; 1981; Kuhn *et al.*, 1980; Kuhn and Lovenberg, 1982; Yamauchi and Fujisawa, 1981; Ehret *et al.*, 1989). Alterations in the activity of CaMKII, and consequently activity of TRP2, could ultimately influence the function of serotonergic neurons by altering the amount of

transmitter available for release into the extracellular space. Evidence therefore implicates CaMKII in the regulation of DA, 5-HT and NA synthesis in the CNS, suggesting that CaMKII may act as a mediator of basal activity for all three transmitters.

It has been well established that CaMKII signaling is critical for the induction of LTP (Pettit *et al.*, 1994; Wang and Kelly, 1995). CaMKII activity and autophosphorylation at the threonine 286 residue are essential for normal NMDA receptor-dependent forms of LTP in the hippocampal CA1 region (Elgersma *et al.*, 2004) and hippocampus-dependent behaviours such as spatial learning and memory (Fink and Meyer, 2002; Lisman *et al.*, 2002; Matynia *et al.*, 2002). Studies with mouse mutants also demonstrate that impairment of αCaMKII signaling severely impairs hippocampus-dependent spatial, as well as contextual, memory formation (Silva *et al.*, 1992; Giese *et al.*, 1998; Elgersma *et al.*, 2004). Using the Morris Water Maze task, αCaMKII autophosphorylation deficient mice take longer to locate a hidden under-water platform compared to wild-types, showing that αCaMKII autophosphorylation is necessary for hippocampal LTP and hippocampus dependent learning (Giese *et al.*, 1998). Subsequent investigation into the specific types of learning and memory to be affected by this αCaMKII autophosphorylation deficiency found that these mice exhibited reduced ‘freezing’ behaviours and were considered to be impaired in cue (amygdala dependent) and contextual (hippocampus dependent) fear conditioning after a single shock (Irvine *et al.*, 2005). Mice were still impaired after three foot shocks in their contextual memory, but not cue fear memory. And after 5 foot shocks, both contextual and cue memories were formed, indicating that these deficits can be overcome by repeated training sessions (Irvine *et al.*, 2005). Data suggest that αCaMKII autophosphorylation affects the speed at which memories are established but not necessarily the capacity to learn. Irvine et al., (2006) conclude that αCaMKII autophosphorylation provides a ‘fast track to memory’ and facilitates learning, but is dispensable for memory.

While there has been a large amount of literature demonstrating the importance of αCaMKII autophosphorylation in learning and memory in rodent

models, until recently there has been no association confirmed in a human sample. Easton and colleagues reported a genetic association between polymorphisms in the human CAMK2A gene and performance in a spatial and non-spatial working memory task in two independent samples (Easton *et al*., 2012). Results suggested that polymorphisms in the human CAMK2A gene predict working memory performance without any corresponding changes in frontal cortex or hippocampal anatomy. This data supports and expands upon findings in animal models which can now be translated to human cognition, thereby strengthening the link between αCaMKII and learning and memory function in both rodents and humans.

Albeit to a lesser extent, emotionality and spontaneous behaviours have previously been examined in CaMKII transgenic mice. αCaMKII KO mice are not as fearful as their heterozygous littermates as they showed reduced freezing behaviours in the foot shock test and reduced defensive and offensive aggression (Chen *et al.*, 1994). Expression levels of αCaMKII seem to play an important role in emotional behaviours (Hasegawa *et al.*, 2009). Transgenic mice which over-express αCaMKII in the forebrain displayed increased anxiety levels and offensive aggression, whereas heterozygous mice showed decreased anxiety and increased defensive aggression (Hasegawa *et al.*, 2009). In addition, stress induced increases in glutamate signalling subsequently lead to increases in the phosphorylation of CaMKII (Suenaga *et al*., 2004). However, it has yet to be determined whether the autophosphorylation mechanism of CaMKII contributes to these behavioural differences in anxiety and aggression. The behaviour of αCaMKII autophosphorylation deficient mice have not been thoroughly characterised. This is surprising, especially given there has been extensive investigation over the past 15 years into the learning and memory deficits of these mice. Moreover, αCaMKII autophosphorylation deficient mice are noticeably more active and more aggressive in response to handling. Mice appear hyperactive, more difficult to capture and attempt to avoid handling by biting and jumping out of the homecage, and displaying enhanced escape behaviours. Taking all evidence into account, there clearly was a need to assess anxiety in αCaMKII autophosphorylation deficient mice. Furthermore, differences in anxiety could

impact upon the more complex test paradigms used to assess drug preference in the current thesis.

Long-term plasticity in the brain requires relatively stable changes in gene expression; these changes may alter neurotransmission and the structure of target neurons (Nestler, 1997; Robinson and Kolb, 2004; McClung and Nestler, 2008). Modifications in αCaMKII expression in the brain have previously been linked to drugs of abuse, lending support to the involvement of CaMKII in pathways of addiction. Alcohol treatment has been shown to induce increases in αCaMKII in prenatal and postnatal rat cerebral cortex (Mahadev *et al.*, 2001), an effect which can be attributed to $Ca^{2+}$ influx during chronic alcohol exposure. However, chronic ethanol treatment has also been shown to have no effect on αCaMKII expression levels in human embryonic kidney (HEK) 293 cells (Xu *et al.*, 2008). It has been previously reported that acute morphine treatment does not affect αCaMKII gene expression (Lou *et al.*, 1999). However, chronic morphine administration was found to induce a specific increase in αCaMKII mRNA levels in the hippocampus and frontal cortex, but not in the amygdala and piriform cortex of the rat brain (Chen *et al.*, 2008). Data illustrate a regional specific regulation of αCaMKII gene expression in response to chronic morphine treatment.

Evidently, αCaMKII autophosphorylation plays a specific role in synaptic plasticity, and particularly in rodent hippocampal and amygdala dependent learning and memory via LTP (Silva *et al.*, 1992; Giese *et al.*, 1998; Elgersma *et al.*, 2004; Irvine *et al.*, 2005). CAMK2A also plays a role in working memory performance in a human sample (Easton *et al*., 2012). Deficiency of αCaMKII autophosphorylation can also potentially alter baseline behaviour and emotionality in mice which needs to be formally assessed. CaMKII expression and consequently long term plasticity have been shown to be mediated by drug administration but the involvement of this autophosphorylation mechanism in the motivational and rewarding effects of drugs of abuse remains unclear. Given the differences in learning and memory tasks and the potential interaction with drugs of abuse, one may speculate as to whether this autophosphorylation mechanism plays an important role in the

motivational and rewarding effects leading to addiction. Since the presence of αCaMKII autophosphorylation can accelerate learning (Irvine *et al.*, 2006), we hypothesised that αCaMKII is more relevant during early drug exposure and the establishment of addiction related behaviours and less so after consumption has been increased. We asked whether the αCaMKII autophosphorylation mechanism may mediate the speed at which addiction related behaviours are established.

### 4.2. Ras-specific guanine nucleotide releasing factor 2 (Ras-GRF2)

The Ras superfamily of proteins includes over 150 GTPases and is made up of six subfamilies, one of which is the Ras subfamily. The Ras subfamily contains 13 members which are split into five subgroups (Ehrhardt *et al.*, 2002). The first group (p21 Ras proteins) consists of the classic Ras proteins (H-Ras, K-Ras and N-Ras) which show considerable homology of 85%. The other four subgroups share approximately 40%–50% homology with the p21 Ras proteins (Ehrhardt *et al.*, 2002). The Ras family of proteins are generally responsible for regulating trafficking and localization in membranes. The proteins play a part in development, growth, differentiation, cell adhesion, proliferation and transport.

Ras-GRF2 belongs to a family of CaM associated guanine nucleotide exchange factors and acts as an ‘on-off switch’ in cells. Ras-GRF2 activates the RAS protein which is known to be involved in the MAPK/ERK signal transduction pathway (Fig. 1.3). The RAS protein activates the MAPK/ERK pathway, by allowing the exchange of Guanosine diphosphate (GDP) for Guanosine-5'-triphosphate (GTP), thereby regulating signalling pathways in cells. NMDA receptor activation of the Ras-MAPK signalling cascade is also critically involved in the induction of LTD and LTP (Thomas and Huganir, 2004; Li *et al.*, 2006). Ras-GRF2 KO mice were impaired in LTP induction, but LTD remained unaffected, demonstrating a specific role in synaptic plasticity (Thomas and Huganir, 2004; Tian *et al.*, 2004; Li *et al.*, 2006; Tian and Feig,

2006). Interestingly, Ras-GRF1 was shown to contribute predominantly to LTD (Li *et al.*, 2006).

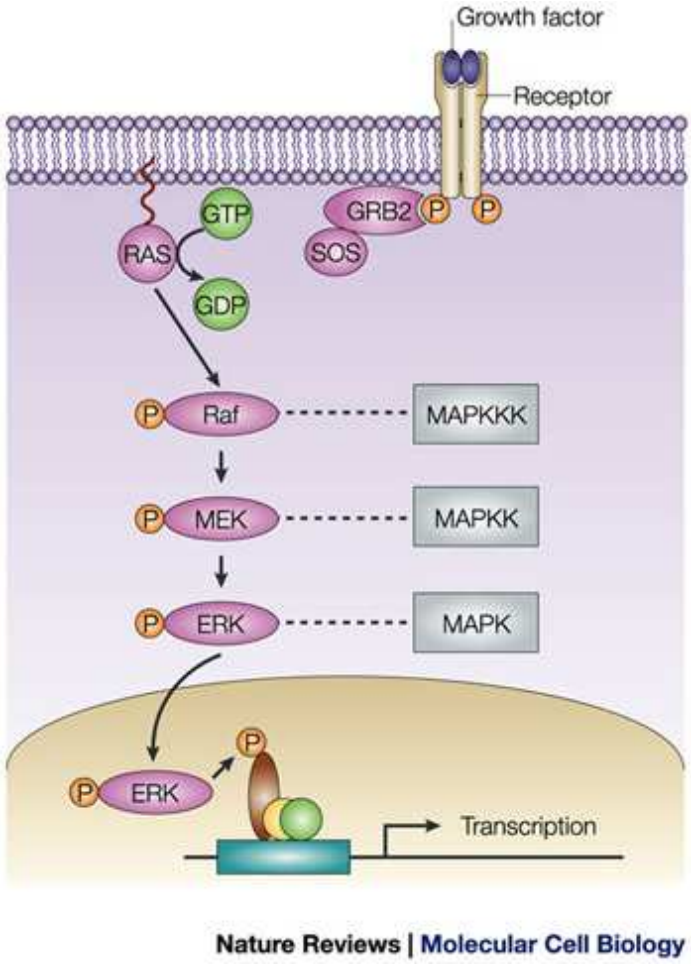


**Figure 1.3: T**he MAPK/ERK signal transduction pathway. The MAPK/ERK signaling cascade has a crucial role in mediating intracellular signal transduction. RAS is activated in the cytosol and then, via this protein kinase cascade, translocates to the nucleus where MAPK regulates the activity of several transcription factors. The cascade begins extracellularly, Epidermal growth factor (EGF) binds to EGF receptor and activates tyrosine kinases (TK) in the cytoplasmic domain of the receptor. Subsequently, intracellular TK's bind docking proteins e.g. GRB2 (Growth factor receptor-bound protein 2) which contain SH2 (Src Homology 2) domains, this in turn binds SOS (son-of-sevenless) which then becomes active, promoting the removal of GDP from RAS. RAS then binds GTP and becomes active itself and activates RAF enzyme. RAF then phosphorylates and activates MEK (MAP kinase kinase) which phosphorylates and activates MAPK. Taken from Kim and Bar-Sagi, 2004.

In many cell types RAS-MAPK/ERK pathway activation promotes cell division. This may explain why mutations in the RAS family of proteins can account for approximately 30% of all human cancers (Bos, 1989). Ras mutations are common in human pancreatic cancer, colorectal cancer, lung adenocarcinoma, gall bladder cancer, bile duct cancer and thyroid cancer (Rowinsky *et al.*, 1999) but not in breast cancer, where Ras mutations have a frequency of less than 5% (Clark and Der, 1995; Rowinsky *et al.*, 1999). For these reasons the RAS-ERK pathway has been a target for a number of anti-cancer drugs and works by inhibiting RAS and other proteins (Hilger *et al.*, 2002). Although deletion of Ras-GRF2 has the potential to have a major impact on neuronal signalling pathways, knockout of Ras-GRF2 does not interfere with basal developmental conditions in mice. Fernandez-Medarde and colleagues (2002) report that Ras-GRF2 is not essential for embryonic and adult mouse development, knockouts have a similar weight and size as their wild-type (WT) littermates, and show similar fertility levels, reaching sexual maturity at the same as WT controls. In addition, no specific pathology was observed following histological analysis. This study demonstrates that Ras-GRF2 is dispensable for mouse growth and development.

Evidence suggests that Ras-GRF2 acts on pathways which are also implicated in alcoholism, including the dopaminergic reward system. The RAS-MAPK/ERK pathway has been linked to DRD1 (Tian *et al.*, 2004; Girault *et al.*, 2007), and has also been isolated as a binding partner of the dopamine transporter (DAT) (Maiya *et al.*, 2007). These studies report a role for Ras-GRF2 both pre- and post-synaptically. Bloch-Shilderman and colleagues (2001) report a direct role for the MAPK/ERK pathway in neurotransmitter release. Ras-GRF2, as a MAPK/ERK activating protein, may therefore be linked to neurotransmitter release. Under basal conditions, alcohol will stimulate neuronal firing in the reward circuitry of the brain. This is mediated in large part by an interaction of alcohol with GABAergic and DA neurons in the ventral tegmental area (McBride *et al.*, 1999). In terminal areas of the DA projections, this typically involves the pre-synaptic neuron becoming depolarized following a large $Ca^{2+}$ influx into the cell. This then sets into

motion a series of events which ultimately result in the release of a given neurotransmitter into the synaptic cleft. Agell and colleagues (2002) report the activation of all Ras-GRF's depend upon $Ca^{2+}$ and Calmodulin. Ras-GRF's contain an ilimaquinone (IQ) motif which dictates $Ca^{2+}$ sensitivity to Ras-GRF. When a cell receives an intracellular signal, the $Ca^{2+}$ channel becomes active, allowing a large $Ca^{2+}$ influx, resulting in depolarization of the cell. Following influx, $Ca^{2+}$ binds to calmodulin forming a (CaM) complex which can in turn bind Ras-GRF2. The $Ca^{2+}$/CaM/Ras-GRF2 complex then activates the RAS protein which induces GDP-GTP exchange, activating the MAPK/ERK pathway and neurotransmitter release. For example, the RAS dependent MAPK/ERK pathway has previously been reported to be involved in DA release in neurons (Bloch-Shilderman *et al.*, 2001). Figure 1.4 represents a simplified version of the proposed mechanism of action suggested here.

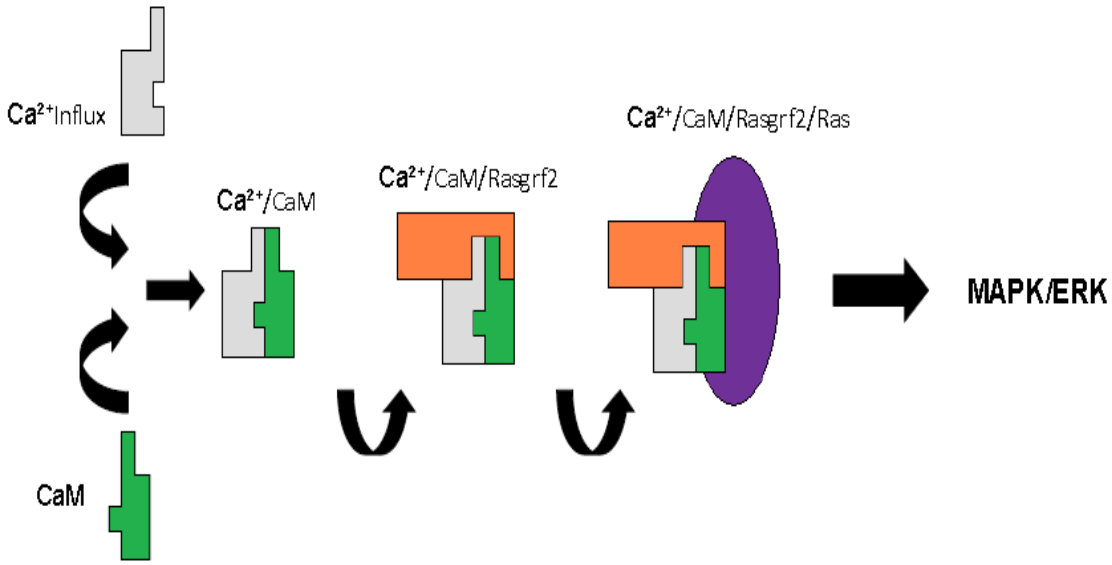


**Figure 1.4**: The $Ca^{2+}$/CaM/Ras-GRF2 complex is responsible for binding to, and activating, the RAS protein. This switches RAS 'on' and subsequently allows RAS to exchange GDP for GTP, resulting in the continuation of the MAPK/ERK pathway and ultimately in neurotransmitter release from neurons.

The potential involvement of Ras-GRF2 in intracellular signalling and neurotransmitter release has considerable implications for the development of drug addiction. Drugs of abuse 'hijack' the natural reward system in the brain (Nestler, 2002b; Kelley, 2004; Hyman, 2005) resulting in the alteration of extracellular transmitter levels in areas such as the NAcc and PFC (Pontieri *et al.*, 1995), thereby mediating the rewarding potential of these drugs (Koob *et al.*, 1998b; Wise, 1998; Swanson, 2000; Nestler, 2001; Everitt and Wolf, 2002). Ras-GRF2 has recently been implicated as a novel candidate in the regulation of alcohol consumption (Stacey *et al*., 2012). Polymorphisms in the human RASGRF2 gene were significantly associated with alcohol consumption, and Ras-GRF2 KO mice also consumed significantly less alcohol and had a reduced alcohol preference compared to controls (Stacey *et* al., 2012). Altogether, evidence from human and animal studies suggests an important contribution of Ras-GRF2 to addiction establishment, in particular for alcohol. Although Ras-GRF2 is a highly speculative candidate for addiction studies, functional mechanisms are currently unknown and warrant further investigation.

## 5. The importance of genetically manipulated animals in behavioural pharmacology research

Humans possess around 30,000 protein coding genes, approximately a third of which have a known function. Trying to discern the function of each gene can be difficult especially since one gene may have several functions, or one function may involve several genes. The use of genetically manipulated animals is becoming increasingly useful when trying to determine single gene function and can be performed in a number of different ways. Targeted insertions work by introducing DNA into embryonic stem (ES) cells, and then cells in which the insert has undergone homologous recombination are selected (Stephens *et al*., 2002). This is comparable to a congenic strain (i.e. where a small region around, and including, a gene is altered) and can result in the elimination ('knock-outs') or alteration ('knock-ins') of gene function. Non-targetted transgenic organisms have had DNA artificially introduced randomly into the genome, resulting in the alteration of normal function of the targeted gene or genetic region. Other types of genetic manipulation include N-ethyl-N-nitrosourea (ENU) mutagenesis which involves the utilisation of a highly potent mutagen (ENU), capable of inducing point mutations within a genome (Russell *et al*., 1979; Noveroske *et al*., 2000; Gondo *et al*., 2010). RNA interference leads to the knockdown or silencing of gene activity by creating double-stranded messenger RNA which is recognised as foreign and destroyed within cells (Lu *et al*., 2005). This process essentially renders the gene inactive. Genetic manipulations have been performed in a variety of organisms including bacteria, yeast, flies, fish, frogs, rodents and even monkeys. These highly sophisticated techniques provide neuroscientists with powerful and, in some cases, specific tools for understanding gene function (Stephens *et al*., 2002).

There are a number of benefits and difficulties when using genetically manipulated animals. The use of such manipulations is highly specific and a major advantage in behavioural pharmacology. By disrupting small selected parts of the genetic sequence distinct behavioural phenotypes may result. Drugs can be used to achieve the same effect as genetic manipulation, however this can have unknown consequences on downstream targets and

may interfere with the hypothesis being tested. Furthermore, the role pharmacokinetics also needs to be considered when assessing pharmacodynamics of the drug under study. Since behavioural pharmacology research routinely involves the use of drugs, the use of genetically modified animals is of considerable benefit. In addition, these techniques provide a way to study certain targets when there are no drugs available to achieve the desired effects. The dopamine D2 receptor (DRD2) is a good example of how genetic techniques can be exploited based on the advantages they possess in terms of specificity. DRD2 naturally occurs in two isoforms, long and short. Deletion of exon 6 of the DRD2 gene results in the expression of the DRD2 short form (Missale *et al*., 1998). This approach allows for the investigation of the two isoforms separately, an effect which cannot be achieved using pharmacological intervention (Glickstein and Schmauss, 2001). Distinct functions of the two isoforms were revealed using a genetic manipulation approach. The DRD2 long form is expressed postsynaptically and is targeted by neuroleptic drugs (Wang *et al*., 2000). The DRD2 short form is expressed presynaptically as an autoreceptor (Usiello *et al*., 2000). The autoreceptor function of the DRD2 short form can control DA release when synaptic concentrations are high i.e. the concentrations achieved during cocaine or morphine treatment (Rouge-Pont *et al*., 2002). The short isoform of DRD2 may therefore be involved in vulnerability to drug abuse.

Genetic manipulation also brings with it some distinct limitations. Insertion of foreign DNA sequences can disrupt function of other genes. This may cause problems with the interpretation of data generated and understanding whether any changes in phenotype are due to the inserted DNA sequence (the congenic footprint effect). It is not uncommon that by introducing mutations at such an early stage (i.e. at the single cell/blastocyst stage), it is not possible to distinguish whether the phenotypic effect observed in the adult is due to an effect of the mutation during development or on the adult. However, the engineering of conditional knockouts deals with both of these issues as the activation of the knockout can be developmental and/or tissue-specific. Genetic background also needs to be given careful

consideration as the effects of mutations can differ according to the strain used (genetic background effect; Gerlai, 1996).

Initially seen as a way to understand behaviour, we can now look at integrating genetic approaches and behavioural pharmacology and investigate how genes influence the mechanisms by which drugs alter behaviour. They help reveal novel systems underlying drug action, which in turn may contribute to understanding the mechanisms of drug abuse and dependence.

## 6. Main research questions, aims and hypotheses

At a cellular level, many drugs of abuse induce a LTP-like state in the DA neurons of the VTA (Bonci et al., 2003; Saal et al., 2003; Thomas and Malenka, 2003; Borgland et al., 2004; Kauer, 2004), LTP is a process which enhances synaptic transmission and potentiates signals for longer within the cell. This is a major cellular mechanism underlying normal learning and memory formation, but is also important for establishing cellular memories of drug exposure and addiction following repeated exposure. The present thesis evaluates whether disruption in key genes of the learning and memory circuitry can affect or alter measures of drug preference, and the rate at which these preferences are established. Specifically, this will be carried out by using an *in vivo* approach to look at the effect of disruption of specific target molecules (αCaMKII and Ras-GRF2) involved in synaptic plasticity on the development of drug preference behaviours. The experiments contained in this thesis assess whether deficits in αCaMKII and Ras-GRF2 can have an impact on the development of drug preference and addiction, and the speed at which these behaviours are established.

**Hypotheses tested in the thesis:**

*Hypothesis 1:*

There is a growing body of research supporting a role for αCaMKII in the behavioural responses to threat or fearful stimuli (Chen *et al.*, 1994; Hasegawa *et al.*, 2009; Suenaga *et al*., 2004). However, the specific contribution of the autophosphorylation mechanism to these behavioural differences remains unknown. From observation alone, it is clear that lack of αCaMKII autophosphorylation alters the behavioural response in novel, potentially threatening, test situations. This is especially important since the internal emotional state has the potential to influence behavioural outcomes used in the succeeding studies. The following hypothesis was tested in Chapter 2:

- αCaMKII autophosphorylation will alter behavioural responses in potentially threatening situations.

The exploratory activity of αCaMKII mutants was tested across a range of novel and familiar test environments. Little is known about the behavioural response of these mice to novel situations and altered anxiety can be a serious confound in the assessment of other behaviours in a novel test situation. This was therefore deemed an important control experiment.

*Hypothesis 2:*

Memory formation and addiction are thought to share a number of molecular and anatomical pathways (Nestler, 2002a; Kelley, 2004; Hyman, 2005; Müller and Schumann, 2011). αCaMKII is important for memory formation (Elgersma *et al.*, 2004) and also for drug addiction (Anderson *et al*., 2008). αCaMKII autophosphorylation deficiency causes learning impairments (Irvine *et al*., 2005), but does not alter the capacity to learn, long term memory storage or memory retrieval (Irvine *et al.*, 2006). This suggests that autophosphorylation may affect the speed of normal learning (Lengyel *et al.*, 2004; Lee *et al.*, 2009). Based on this evidence, the following hypotheses were tested in Chapters 3 and 4:

- The lack of autophosphorylation should lead to a delay in the establishment of addiction related behaviours.
- This should not limit the capacity to establish these behaviours after prolonged drug exposure, or play a role in the maintenance of an addictive state once established.
- The initial delay should coincide with changes in the monoaminergic system

Alcohol increases αCaMKII expression levels in rat cerebral cortex which can be attributed to $Ca^{2+}$ influx during chronic alcohol exposure (Mahadev *et al.*, 2001). Chapter 3 therefore tests the above hypotheses in relation to alcohol use. Also, cocaine reinstatement is associated with CaMKII activation and phosphorylation of CaMKII at the Thr286 site (Anderson *et* al., 2008). Chapter

4 therefore investigates the involvement of cocaine relative to the above hypotheses.

*Hypothesis 3:*

Ras-GRF2 is a novel candidate in the regulation of alcohol consumption (Stacey *et al*., 2012). Polymorphisms in the human RASGRF2 gene are significantly associated with alcohol consumption, and Ras-GRF2 KO mice consume and show a reducd preference for alcohol compared to controls (Stacey *et* al., 2012). Ras-GRF2 KO mice are impaired in LTP induction, demonstrating a specific role for this gene in synaptic plasticity (Thomas and Huganir, 2004; Tian *et al.*, 2004; Li *et al.*, 2006; Tian and Feig, 2006). The RAS-MAPK/ERK pathway has been linked to the dopaminergic system (Tian *et al.*, 2004; Girault *et al.*, 2007; Maiya *et al.*, 2007) and neurotransmitter release (Bloch-Shilderman *et al*., 2001). This has potential implications for the development of drug addiction. Chapter 5 aims to further examine the reason for diminished alcohol drinking behaviours in Ras-GRF2 KO mice. The following hypotheses were tested:

- Ras-GRF2 KO mice do not experience the motivationally rewarding properties of alcohol to the same extent as wild-type mice.
- This may be accounted for by dysfunction of neurochemistry in the reward system.

Evidence from human and animal studies suggests an important contribution of Ras-GRF2 to addiction establishment, for alcohol in particular. However, functional mechanisms are currently unknown and warrant investigation. Ras-GRF2 is a highly speculative candidate for addiction studies. However, further examination may provide evidence for a potential link between this gene and addiction.

It is important to note that any consequences of the manipulation of either gene on behaviours related to addiction are not necessarily attributable to their roles in learning. Experiments performed in the thesis do not explicitly test learning and memory in these mice. Further experimentation would be required in order to definitively determine whether it is the learning deficit in

these animals causing any alterations seen in addictive behaviours. Data from this thesis merely suggests an association between αCaMKII and Ras-GRF2 and addiction related behaviours, whether this is mediated by altered LTP/learning mechanisms necessitates further investigation.

# αCaMKII autophosphorylation controls exploratory activity to threatening novel stimuli

Chapter 2

**Easton, A. C.,** W. Lucchesi, G. Schumann, K. Peter Giese, C. P. Müller and C. Fernandes (2011). "αCaMKII autophosphorylation controls exploratory activity to threatening novel stimuli." Neuropharmacology 61(8): 1424-31.

## Abstract:

Autophosphorylation of αCaMKII is regarded as a 'molecular memory' for $Ca^{2+}$ transients and a crucial mechanism in aversely, but less so in appetitively, motivated learning and memory. While there is a growing body of research implicating αCaMKII in general in behavioural responses to threat or fearful stimuli, little is known about the contribution of the autophosphorylation. The present study asked how αCaMKII autophosphorylation controls anxiety-like behavioural responses toward novel, potentially threatening stimuli. Homozygous and heterozygous T286A αCaMKII autophosphorylation deficient mice and wild types were tested in a systematic series of behavioural tests. Homozygous mutants were more active in the open field test and showed reduced anxiety-related behaviour in the light/dark test, but these findings were confounded by a hyperlocomotor phenotype. The analysis of elevated plus maze showed significantly reduced anxiety-related behaviour in the αCaMKII autophosphorylation-deficient mice which appeared to mediate a hyperlocomotor response. An analysis of home cage behaviour, where neither novel nor threatening stimuli were present, showed no differences in locomotor activity between genotypes. Increased locomotion was not observed in the novel object exploration test in the αCaMKII autophosphorylation-deficient mice, implying that hyperactivity does not occur in response to discrete novel stimuli. The present data suggest that the behaviour of αCaMKII autophosphorylation-deficient mice cannot simply be described as a low anxiety phenotype. Instead it is suggested that αCaMKII autophosphorylation influences locomotor reactivity to novel environments that are potentially, but not necessarily threatening.

## 1. Introduction:

There is a growing body of research supporting a role for αCaMKII in behavioural responses to threat or fearful stimuli. Chen and colleagues (1994) show that αCaMKII knockout mice exhibit reduced freezing behaviour in the foot shock test and, therefore, do not appear to be as fearful as their heterozygous littermates. Homozygous (KO) mice also displayed reduced defensive and offensive aggression. This is interpreted as a sign of reduced fear in the KO mouse (Chen *et al.*, 1994). A recent study generated transgenic mice over-expressing αCaMKII in the forebrain and reports increased offensive aggression and increased anxiety levels in these mice (Hasegawa *et al.*, 2009). Suenaga and colleagues (2004) demonstrate that stress increases glutamate signalling, which in turn increases the phosphorylation of CaMKII. Also, expression levels of αCaMKII play an important role in emotional behaviours (Hasegawa *et al.*, 2009). However, it remains unclear how important the specific role of the autophosphorylation mechanism contributes to these behavioural differences.

Prior to commencing the present study, mice were seen to react with unusually high levels of aggression or fear towards the experimenter, exhibiting behaviours such as biting and responding in a very hyperactive way in an attempt to escape. This raised the question of differential threat reactivity and anxiety-like responses present in the αCaMKII autophosphorylation deficient mice. The behaviour of these mice has never been thoroughly characterised. This is surprising, especially since there has been extensive investigation into the learning and memory deficits of these mice. Any differences in basal behavioural responses have the potential to interfere with further behavioural paradigms. αCaMKII autophosphorylation deficient mice show learning deficits in tasks associated with aversive stimuli or a stressful or emotional component (Giese *et al*., 1998; Need and Giese, 2003; Irvine *et al*., 2005), but not in appetitively motivated tasks (Carvalho *et al*., 2001). Spontaneous behaviour tests assess anxiety-like traits in response to anxiolytic drugs, such as benzodiazepines. More recently, these tests have been used to assess basal behavioural differences between mice, for

example, between mice of different strains or genotypes. Tests such as the open field, light/dark box, elevated plus maze, homecage, and novel object have a high sensitivity to anti-anxiety pharmacological agents. Moreover, these tests are based on the monitoring of spontaneous behaviours, which is a major strength of the test battery in the present study. This provides a high degree of ethological validity, relying upon unconditional reactions to potentially threatening novel situations.

In their book 'The Neuropsychology of Anxiety' (2000), Gray and McNaughton analyse the cognitive and behavioural functions of anxiety in the septo-hippocampal system and the roles of these functions in memory and fear. There is a distinct difference between fear and anxiety. Fear implies immediate threat, whereas anxiety suggests threat is possible although not yet present. The theory suggests that this is mainly controlled by the septo-hippocampal system and its connections to other brain regions such as the amygdala (Gray and McNaughton, 2000). The system is designed to detect conflict between competing goals (such as food, threat, escape, etc.). Anxiety manifests when there is competition between two goals which are controlled by the septo-hippocampus. This conflict creates uncertainty, and the hippocampus tries to resolve this through behavioural inhibition i.e. approach or avoidance. The hippocampus has been studied extensively as part of the brain system responsible for spatial memory (O'Keefe and Nadel, 1978; Silva et al., 1992; Giese et al., 1998; Fink and Meyer, 2002; Lisman et al., 2002; Matynia et al., 2002; Elgersma et al., 2004). Lesions of the hippocampus impairs spatial memory as measured in the Morris Water Maze task (Morris *et al*., 1982). Gray and McNaughton (2000) argue that such a lesion would also remove the inhibitory system which attempts to resolved goal conflict. Therefore, does destruction of the hippocampus cause spatial deficits or simply an inability of the system to resolve goal conflict i.e. trying to escape versus interpretation of sensory cues. This is described as a hierarchical defense system theory that relates anxiety, fear and memory.

Differences in cognitive processing have the potential to alter resulting emotional responses (McNaughton, 1997). Furthermore, cognitive systems

have been described as a filter for environmental information in order to produce an appropriate emotional response (McNaughton, 1997). The limbic system, and the hippocampus in particular, has been identified as crucial for both memory (O'Keefe and Nadel, 1978) and emotion (Gray, 1992). It therefore follows that there is potential for overlap between the two processes, where cognitive dysfunction and increased negative associations of stimuli can result in increases in anxiety (McNaughton, 1997).

Given the differences in emotion-related behaviours observed in these mice and the potential interaction between anxiety-like behaviours and cognition (Gray and McNaughton, 2000), the aim of the current study is to investigate how αCaMKII autophosphorylation controls behavioural responses towards potentially threatening situations.

## 2. Material and Methods

All housing and experimental procedures were performed in accordance with the U.K. Home Office Animals (Experimental Procedures) Act 1986.

### 2.1. Animals

Male and female αCaMKII$^{T286A}$ mice (Giese *et al.*, 1998) were studied. These mutants were generated using a gene-targeting strategy which utilizes a replacement vector containing the point mutation and a neo gene flanked by loxP sites (Fig 2.1); after homologous recombination the neo gene was removed by Cre recombination (Giese *et al.*, 1998). R1 ES cells (F1 between 129/Sv and 129/Sv-CP) were used. An initial PCR was used to detect the loxP site which determining the genotype. A second PCR was used to identify the point mutations. The mice were subsequently backcrossed for at least eight generations into C57BL/6J and then crossed once with 129S2/SvHsd mice and kept in this mixed background by interbreeding for reasons of fecundity. (Mice had gone through more than four rounds of interbreeding before testing). Mice were obtained from interbreeding of heterozygotes and genotypes were determined by PCR with tail biopsies as described (Irvine *et al.*, 2005). In the homozygous mutants the autophosphorylation of αCaMKII is prevented due to the insertion of a missense mutation (threonine-286 changed to alanine; T286A) within the autoinhibitory domain (Giese *et al.*, 1998). This mutation does block the autophosphorylation of CAMKII but does not affect the $Ca^{2+}$ dependent activity (Giese *et al.*, 1998). Animals were provided with food and water ad libitum, and kept on a 12:12 h light: dark cycle (lights on at 7.00 am). Behavioural tests were performed during the light cycle between 09:00 and 16:00 h. Room temperature was maintained between 19 ℃ and 22 ℃ at a humidity of 55% (±10%) . Animals were singly housed when adult to avoid any potential confounds from social hierarchies and aggressive behaviour 1 week prior to behavioural testing. Mice were housed in Tecniplast cages (32 cm x16 cm x 14 cm), using Litaspen sawdust and nesting materials, (Sizzlenest, Datsand, Manchester UK).

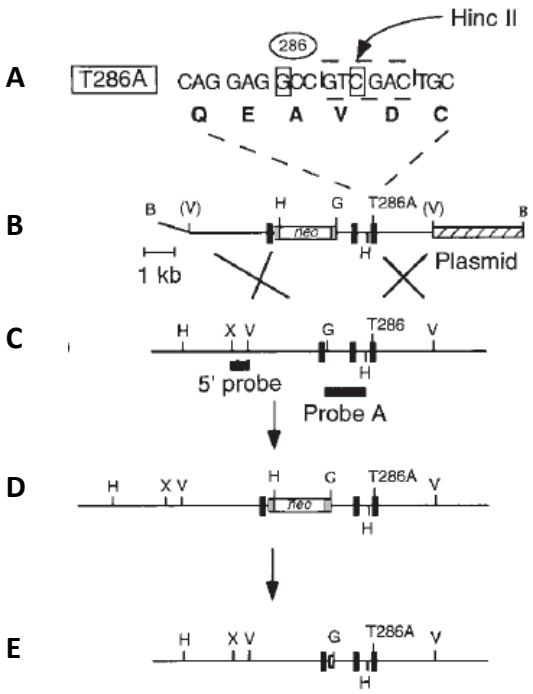


**Figure 2.1:** Generation of the αCaMKII$^{T286A}$ mutants with the Pointlox procedure. (A) The targeting construct (B), a partial map of the αCaMKII gene (C), the resulting targeted allele (D), and the targeted allele after Cre recombination (E) are illustrated. B, Bam HI; G, Bgl II; H, Hind III; V, Pvu II; X, Xba I. Taken from Giese *et al.*, 1998.

## 2.2. Experimental procedures

Male and female wild type (WT), homozygous (Mt) and heterozygous (Ht) αCaMKII$^{T286A}$ mice were tested through a battery of behavioural tests in the following order: open field, light-dark box, elevated plus maze, home-cage activity and novel object exploration. Group sizes were the same for open field, light-dark box, elevated plus maze and novel object exploration, (WT n=5 (M=3, F=2); Ht n=7 (M=5, F=2); Mt n=7 (M=3, F=4)). For the home-cage test a different batch of mice was used which had not previously been exposed to potentially threatening situations and stimuli (WT n=8; Ht n=8; Mt n=8; M:F ratio was 50:50 in each group). Mice were excluded from the test if they either escaped from the apparatus or were completely inactive (open field = 1 WT female; Light-dark box = 1 Ht female; elevated plus maze = 2 Mt males). Mice were tested in a pseudorandom order and were moved to the

behavioural suite adjacent to the housing room immediately before testing. Apart from the homecage test, each test apparatus was wiped clean with 1% Trigene® between subjects to avoid any olfactory cues influencing behaviours. Mice were returned to their home cage at the end of each test and allowed to recover for at least five days before further testing. Behaviours for all tests were recorded on videotape for subsequent scoring by an experimenter blind to the genotype of the mice.

*2.2.1. Experiment 1: Open Field*

Each mouse was placed in a square white acrylic arena (72×72×33 cm), facing an outer wall, for 10 min and allowed to freely explore the arena (Fig. 2.2). White light of 25 lx was evenly distributed across the arena during testing (Hall, 1921). Video recordings were taken using EthoVision and analyzed using the automated tracking software (Noldus *et al.*, 2001; Spink *et al.*, 2001); Fig. 2.2B**)**. A virtual square of equal distance from the periphery (36×36 cm) was defined as the 'central zone' in order to determine the number of entries, and time (s) spent in the central zone (Fig. 2.2A). Locomotor activity in the outer and inner zones, distance moved (cm) and mean speed (cm/s) were measured.

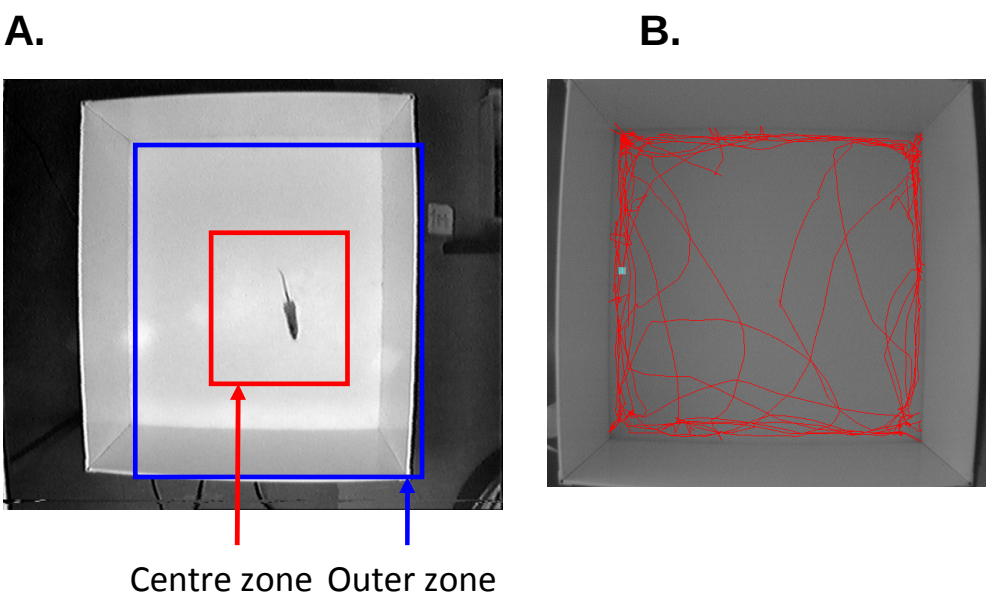


**Figure 2.2:** Open Field experimental set-up.

*2.2.2. Experiment 2: Light/Dark Test*

For the light-dark test (Crawley and Goodwin, 1980), a box of white acrylic was used (44×21×21cm) (Fig. 2.3). The box was partitioned with a white acrylic wall, so that approximately one-third (15×21×21 cm) of the total area was dimly lit (15-20 lx) and therefore represented the 'dark chamber'. The remaining two thirds were brightly lit (80-110 lx) with white light, which served as the 'light chamber'. A small entry door within the partition (5×7 cm), allowed mice to move freely between chambers. Each mouse was taken from its home cage and placed into the dark chamber facing the end wall parallel to the partition. Activity was recorded for the following 5 minutes using EthoVision. Locomotor activity (distance moved in cm, mean speed in cm/s), transitions between, and the time spent in both the dark and light chambers were measured. A single transition was counted when two paws had entered a chamber.

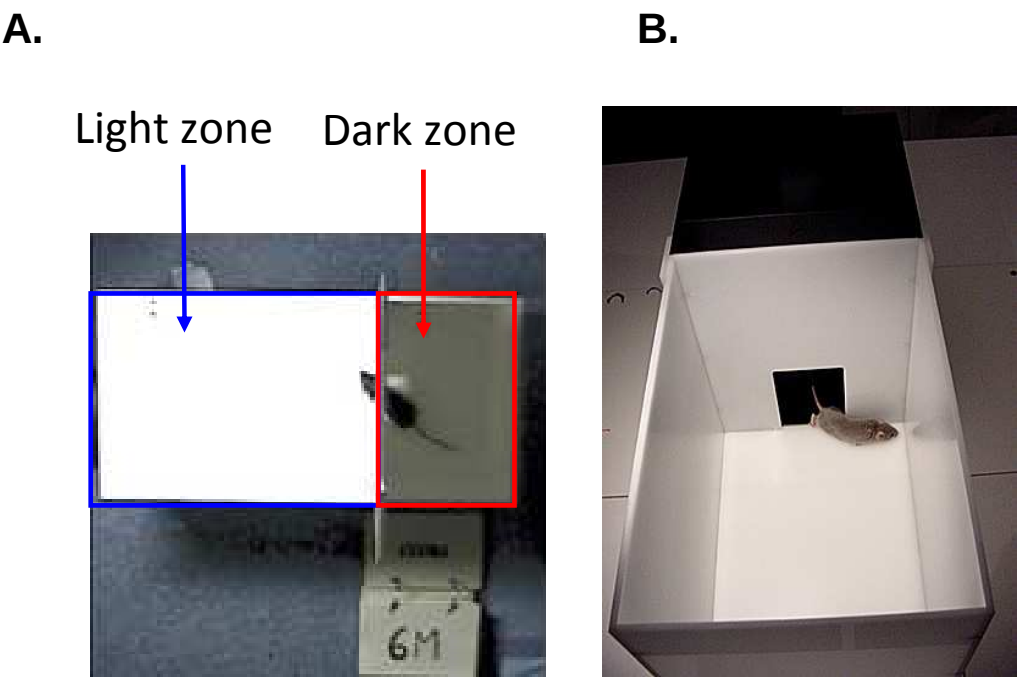


**Figure 2.3:** Light/Dark Test experimental set-up

*2.2.3. Experiment 3: Elevated Plus Maze*

The elevated plus maze (Lister, 1987) was constructed from black opaque acrylic, each arm measuring 30×5 cm and the central platform 5×5 cm (Fig. 2.4). One set of arms, opposing one another, were enclosed completely by a wall of opaque acrylic, 15 cm high, while the other set was open with a ledge of 0.5 cm either side of the arms. The maze was elevated 40 cm from the ground on a transparent acrylic stand. The mouse was placed on the central platform, facing towards a closed arm, and allowed to freely explore the maze for 5 min. EthoVision tracking software was used to record locomotor activity during the test (mean speed in cm/s and distance moved on the open and closed arms), and the number of entries into, and time spent on, closed and open arms. An arm entry was counted when two paws had entered an arm, and an arm exit was determined when two paws had left the arm.

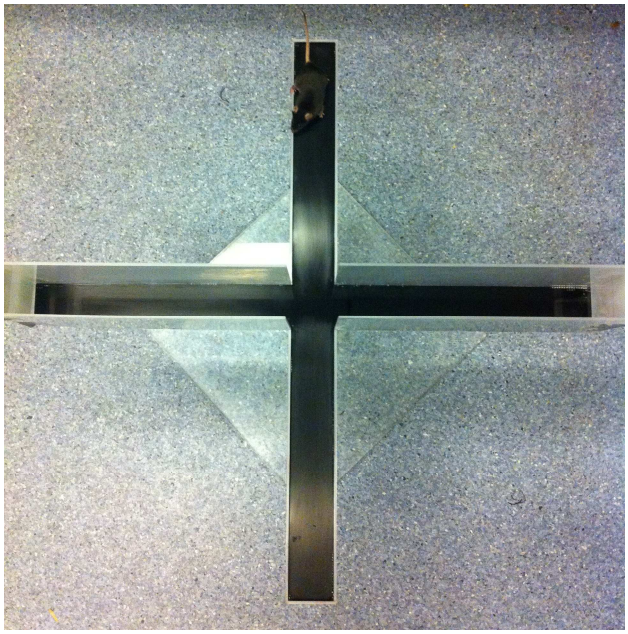

**Figure 2.4:** Elevated Plus Maze experimental set-up

*2.2.4. Experiment 4: Homecage*

This task was run in the homecage (32x16x14 cm) in order to observe spontaneous behaviours in a familiar environment (Mill *et al.*, 2002); Fig. 2.5). The test room light intensity and light cycle matched that of the housing room. All nesting materials were removed from the cages. Cage tops were replaced with a Plexiglas lid (42×25.5 cm) to prevent escape. Activity was recorded for two hours. Video recordings were analyzed using the automated EthoVision tracking software (Noldus *et al.*, 2001; Spink *et al.*, 2001), from which total distance moved (cm) and mean speed (cm/s) were derived.

**Figure 2.5:** Homecage experimental set-up

*2.2.5. Experiment 5: Novel Object Exploration*

The novel object exploration paradigm (Dere *et al.*, 2007) was used to detect differences in exploratory behaviour between groups in a familiar arena, in the presence of novel, potentially non threatening stimuli (Fig. 2.6). The test room was evenly lit with low white light, at approximately 25 lx. Each mouse was taken from its home cage and briefly placed into a clean, temporary cage. Two identical glass (8x8x5.5 cm; Fig 2.6A.) or plastic (10x5x7 cm; Fig 2.6B.) objects were introduced into the homecage, counter-balanced across genotype to avoid any object preference confound. Two identical objects were used to attempt to maximize exploration but avoid any preference between these objects (Dere *et al.*, 2007). The animal was quickly reintroduced in the homecage, facing an outer wall. Cage tops were replaced with a Plexiglas lid (42×25.5 cm) to prevent escape. The mouse was allowed to explore the objects freely for 10 minutes whilst activity was tracked using EthoVision software. The time (s) spent in object exploration was scored manually to specifically measure directed exploration towards the objects. Total Distance moved (cm), mean velocity (cm/s) and time spent exploring each object (s) were measured.

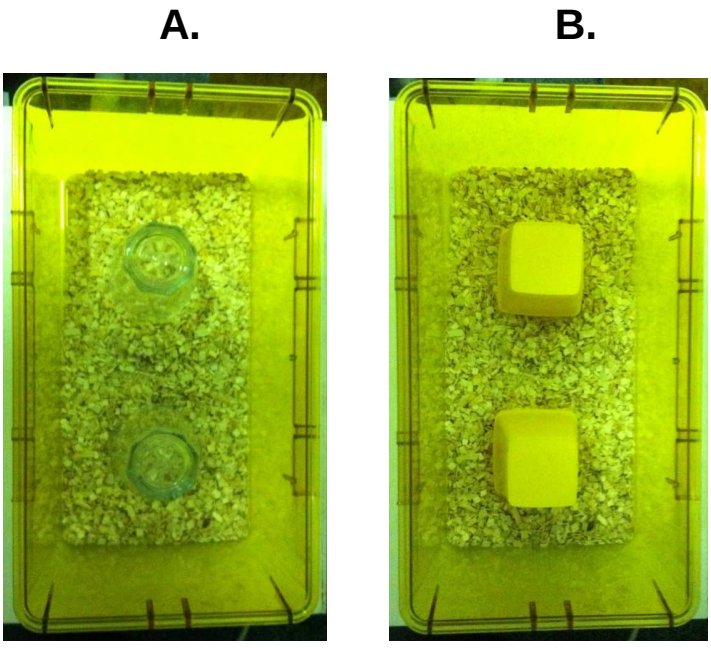


**Figure 2.6:** Novel Object Exploration experimental set-up

**2.3. Statistical Analysis:**

All graphical output data is expressed as a mean ± SEM. Data were initially compared using a two-way analysis of variance (ANOVA) using factors genotype (3) and sex (2) to assess potentially confounding sex differences. Data were subsequently compared using one-way ANOVA to assess group differences. Tukey's HSD test was used post-hoc to determine the differences between individual group means. Analysis of Co-Variance (ANCOVA) was used to determine whether there was an interaction effect between confounding measures of locomotor activity and anxiety. Planned pair-wise comparisons (independent by variable t-tests) were performed between certain trials for each genotype group. A significance level of $p<0.05$ was used to test for statistical significance. The software used was Statistica version 7.1.

When designing animal experiments, the sample size is typically selected based on previous literature. However, this can often result in an overestimation of the animal numbers needed, which can mean a waste of resources and unnecessary animal suffering. On the other hand, an underestimate of group size means that the sample size may be too small to achieve the scientific objectives of the study (Festing *et al*., 2002). Sample size can be estimated using either a power analysis or application of the resource equation method. As a general rule, the power analysis method is the most appropriate for relatively simple but extensive experiments which are likely to be repeated several times with slightly different treatments. The resource equation method is more suitable for complex biological experiments involving several treatment groups, quantitative data, and where the standard deviation cannot be estimated (Festing *et al*., 2002).

The present study retrospectively used the 'Resource Equation' (Mead, 1988) method to quantify the probability that experiments detected biologically important effects. Mead's resource equation (not using blocking) states:

**E = (total number of experimental units) – (number of treatment combinations*)**

*Treatment combinations = 2 (sexes) x 3 (genotypes) x 1 (doses)

**E** was calculated for all experiments in the present study. Open Field, **E** = 13; Light/Dark, **E** = 13; Elevated Plus Maze, **E** = 13; Homecage, **E** = 18; Novel Object Exploration, **E** = 13. According to Mead's rule, **E** should be between 10 and 20. If **E** is less than 10, increasing numbers would lead to good returns. If **E** is over 20, resources may be wasted.

# 3. Results

All data were initially analysed using a two-way ANOVA for factors genotype and sex (Crusio *et al.*, 2009). There was no significant effect of sex in any of the tests used and consequently the data for males and females were combined for subsequent analyses.

## 3.1. Experiment 1: Open Field

An open field test was performed in order to test locomotor activity and anxiety-like behaviour in a novel environment. ANOVA revealed a significant effect of genotype on the distance moved in the outer zone of the arena ($F(2, 15) = 9.53$, $p < 0.01$; Fig. 2.7b). Post-hoc Tukey test revealed that αCaMKII autophosphorylation-deficient (Mt) mice tended to move more in the outer zone of the arena compared to wildtype mice ($p=0.053$) and moved significantly more than heterozygous mutants (Ht) ($p<0.001$). Genotype had a significant effect on average speed in the outer zone ($F(2, 15) = 11.18$, $p<0.001$; Fig. 2.7d). Mt mice were found to move faster than both wild-type (WT) ($p<0.01$) and Ht ($p<0.001$) mice. Genotype also had a significant effect on average speed in the centre zone ($F(2, 15) = 8.8102$, $p<0.01$; Fig. 2.7c). As with speed in the outer zone, Mt mice were hyperactive compared to WT ($p<0.05$) and Ht ($p<0.01$) mice. Since there is no significant genotypic difference for the number of entries made (Fig. 2.7e), the distance moved (Fig. 2.7a) and time spent in the centre zone (Fig. 2.7f), but a significant trend for increased distance in the outer zone and speed moved in the outer and centre zones, results suggest that the Mt have increased locomotor activity compare to the other genotypes.

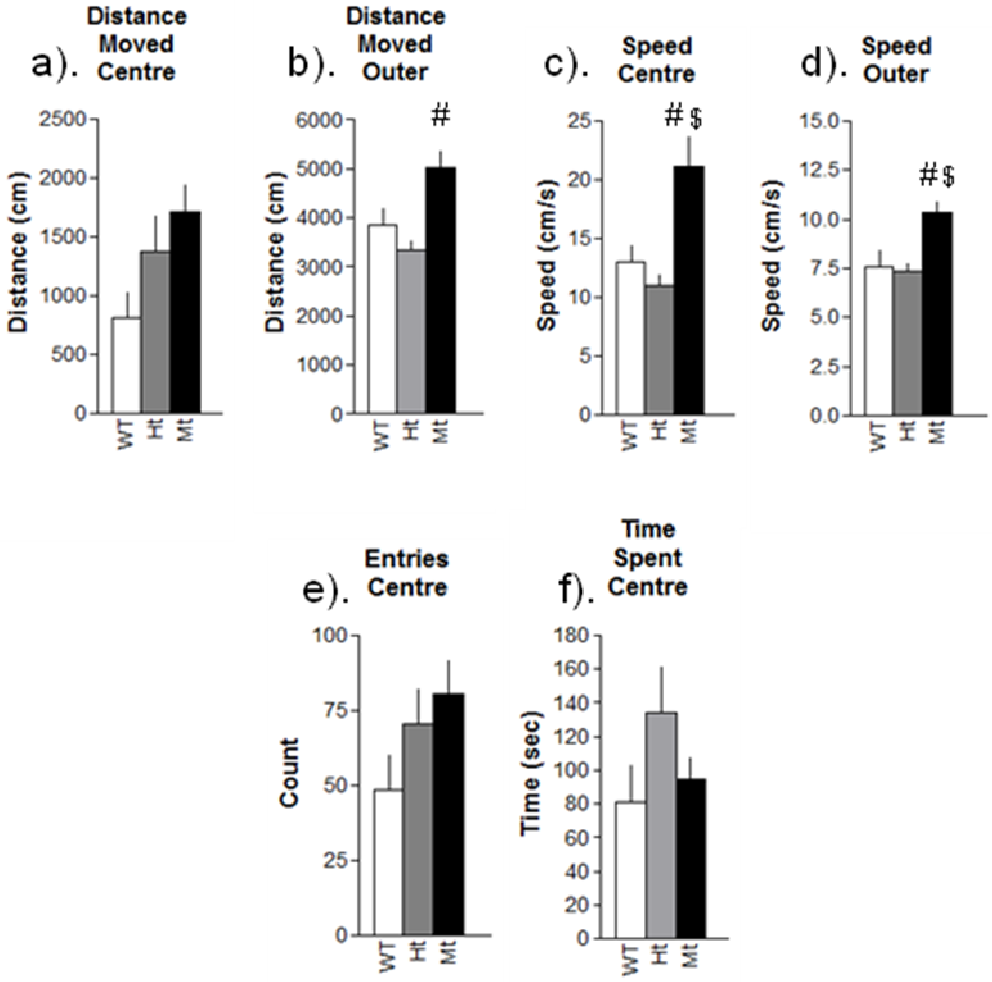


**Figure 2.7:** The effects of αCaMKII autophosphorylation deficiency on open field behaviour. Bars represent mean values ± standard error of the mean (SEM). Post-hoc Tukey HSD test significance: $^{\$}$ $p<0.05$, Tukey HSD test vs. WT. # $p<0.05$, Tukey HSD test vs. Ht.

### 3.2. Experiment 2: Light/Dark Test

The light/dark test showed no difference in the distance moved between groups in the dark compartment (Fig. 2.8a). There was however a genotype effect on the distance moved in the light compartment ($F(2, 15) = 10.12$, $p<0.01$; Fig. 2.8b), between Mt and both WT ($p<0.05$) and Ht ($p<0.01$) mice. Fig. 2.8c illustrates a difference in time spent in the dark ($F(2, 15) = 4.86$, $p<0.05$), seen between Mt and Ht mice ($p<0.05$) and as a tendency between Mt and WT ($p=0.053$). Furthermore, there was a significant effect of genotype regarding time spent in the light area ($F(2, 15) =4.93$, $p <0.05$; Fig. 2.8d) with Mt spending more time in the light area than WT ($p<0.05$) and Ht mice ($p<0.05$). The average speed in the dark compartment differed significantly ($F(2, 15) = 9.42$, $p<0.05$; Fig. 2.8e). Mt mice moved faster in the dark compartment compared to WT ($p<0.01$) and Ht ($p<0.01$) mice. Speed in the light compartment was also affected by genotype ($F(2, 15) = 4.27$, $p<0.05$; Fig. 2.8f), but only in Mt compared to Ht mice ($p<0.05$). In addition to these findings, the number of transitions between the two zones differed significantly between the genotypes ($F(2, 15) = 10.45$, $p<0.001$; Fig. 2.8g). Mt made more transitions than WT ($p<0.01$) and Ht mice ($p<0.01$), suggesting increased risk assessment behaviours in the αCaMKII mutant mice. Given the differences in activity and the time spent in the light area observed in the Mt group, an analysis of co-variance (ANCOVA) was conducted to assess which behavioural measure was contributing the most to the variance in the observed behaviour. Speed in the dark was used as a measure of locomotor activity, and time in the light was used as an anxiety trait. ANCOVA results showed a significant interaction effect ($F(2, 14) = 39.6$, $p<0.001$), but when each behavioural measure was individually co-varied out, the results were no longer significant, indicating that the behaviours were confounded and dependent on one another. The results of the light/dark test may superficially suggest a reduced anxiety in αCaMKII autophosphorylation-deficient mice in this novel environment. However, they also show a significantly enhanced locomotor response to the novel situation, so that genotype effects on anxiety and general locomotor activity could not be fully segregated at this level.

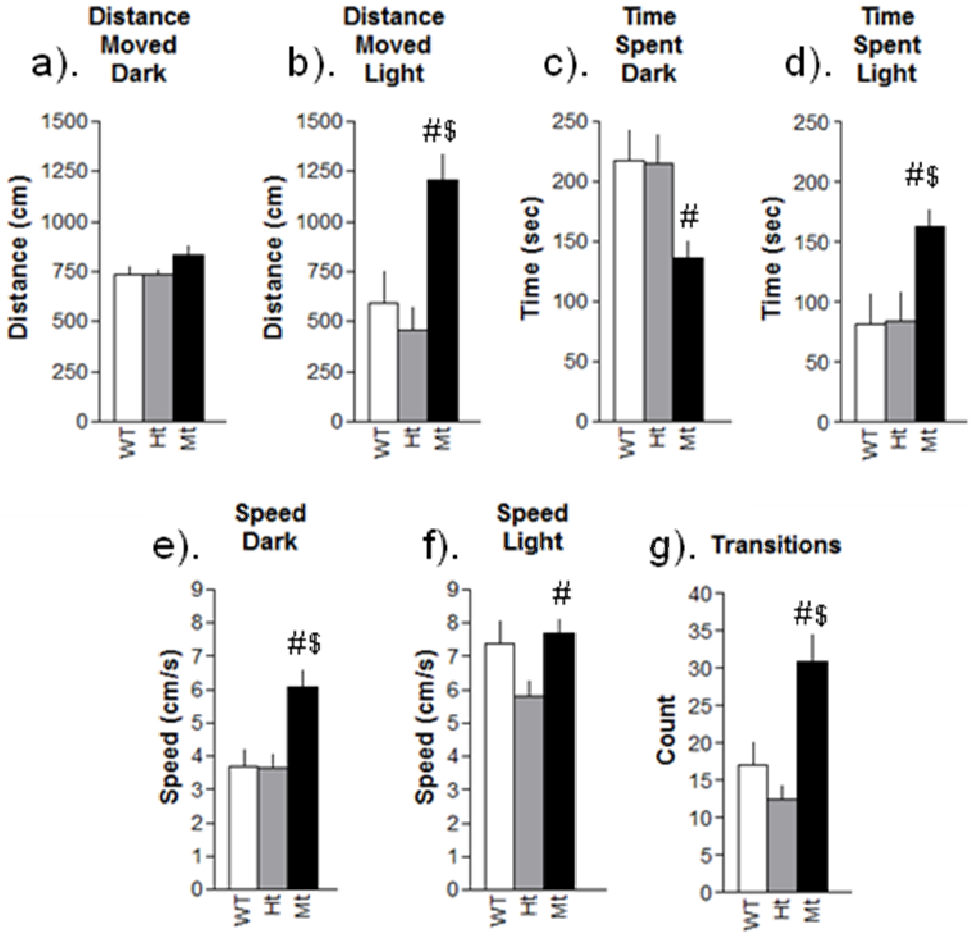


**Figure 2.8:** The effects of αCaMKII autophosphorylation deficiency on behaviour in the Light/Dark test. Bars represent mean values ± standard error of the mean (SEM). Post-hoc Tukey HSD test significance: $ p<0.05, Tukey HSD test vs. WT. # p<0.05, Tukey HSD test vs. Ht.

### 3.3. Experiment 3: Elevated Plus Maze (EPM)

The time spent on the open arms of the EPM was significantly different between the genotypes ($F(2, 14) = 58.19$, $p<0.001$; Fig. 2.9a); Mt differed from both WT ($p<0.001$) and Ht mice ($p<0.001$). There was also an opposite effect of genotype on time spent in the closed arms ($F(2, 14) = 38.58$, $p<0.001$; Fig. 2.9b), with Mt mice spending less time in the closed arms than WT ($p<0.001$) and Ht mice ($p<0.001$). The genotype had a significant effect on the number of entries into the open arms of the maze ($F(2, 14) = 26.46$, $p<0.001$). Mutant mice made more entries than WT ($p<0.001$) and Ht ($p<0.001$) mice (Fig. 2.9c). This difference was not found for groups in the number of closed arm entries made (Fig. 2.9d) or distance moved in the closed arms (Fig. 2.9h). A significant difference was seen between groups in the average speed of movement ($F(2, 14) = 25.28$, $p<0.001$; Fig. 2.9e) and distance moved ($F(2, 14) = 86.4539$, $p<0.001$; Fig. 2.9g) in the open arms of the maze. Mt moved significantly faster than both WT ($p<0.001$) and Ht mice ($p<0.001$), and Mt mice also moved significantly greater distances than WT ($p<0.001$) and Ht ($p<0.001$) mice. The same effect was seen between groups in the closed arms of the EPM ($F(2, 14) = 10.21$, $p <0.01$; Fig. 2.9f). Mt moved at a faster speed than WT ($p<0.01$) and Ht mice ($p<0.01$). These results confirm the hyperactive phenotype observed in the absence of αCaMKII autophosphorylation. To assess whether locomotor activity and anxiety measures were confounded in the EPM, an ANCOVA was performed to determine which behaviour (time in the open arms vs. speed in the closed arms) was contributing the most to the variance observed in the behaviour. The ANCOVA produced a significant interaction effect ($F(2, 13) = 18.37$, $p <0.001$), a result which retained significance when co-varying out locomotor activity ($F(2, 13)=18.4$, $p <0.001$). In contrast, when accounting for anxiety differences in the mice, the locomotor measure was no longer significant ($F(2, 13)= 0.06$, $p=0.94$). This indicates that anxiety was influencing the locomotor activity in this test and suggests that Mt mice have essentially a reduced anxiety-related behaviour rather than a hyperactive phenotype in the EPM.

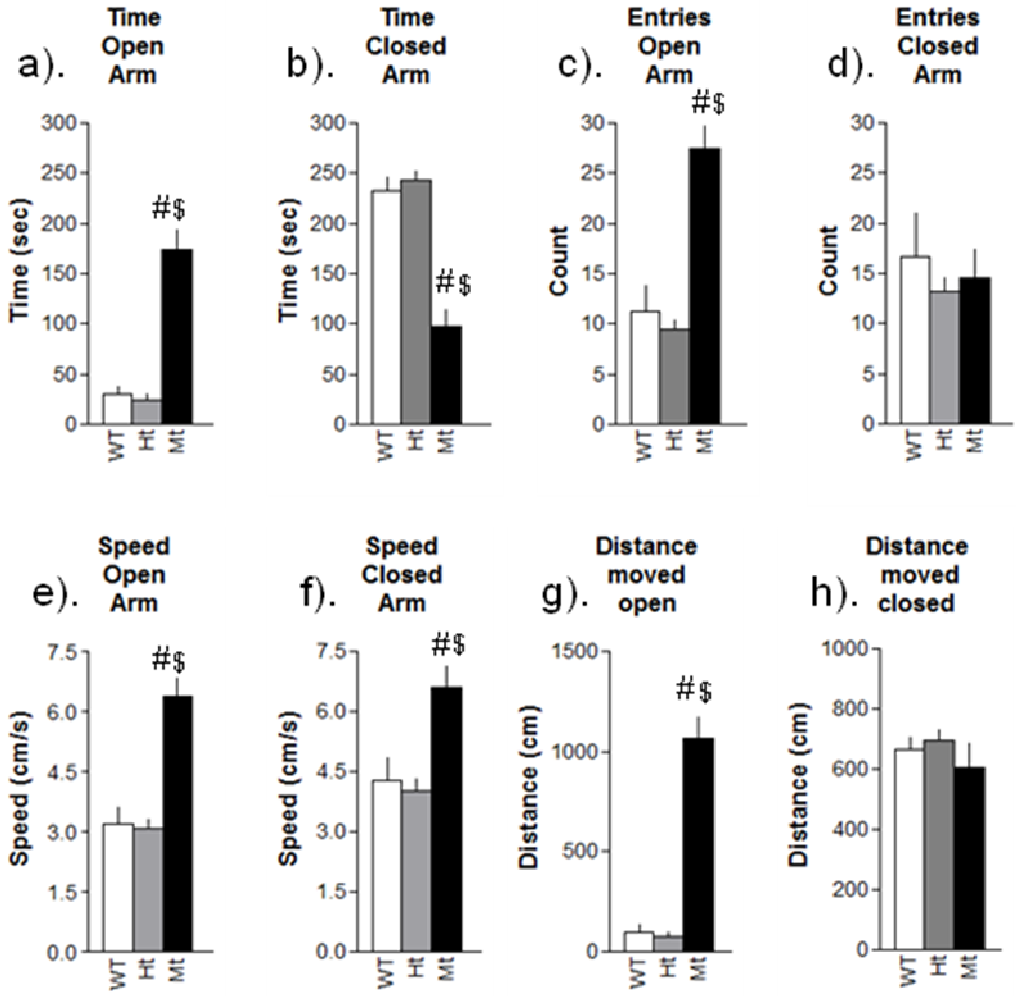


**Figure 2.9:** The effects of αCaMKII autophosphorylation deficiency on behaviour in the Elevated Plus Maze. Bars represent mean values ± standard error of the mean (SEM). Post-hoc Tukey HSD test significance: $ $p<0.05$, Tukey HSD test vs. WT. # $p<0.05$, Tukey HSD test vs. Ht.

### 3.4. Experiment 4: Homecage

The homecage test provided a measure of spontaneous activity in a familiar environment. There were no significant differences in the total distance moved (Fig. 2.10a) or average speed (Fig. 2.10b) between the genotype groups during the two hour period ($p>0.05$). This suggests that the Mt mice are hyperactive only in novel, potentially threatening environments, but not in familiar and/or non-threatening environments.

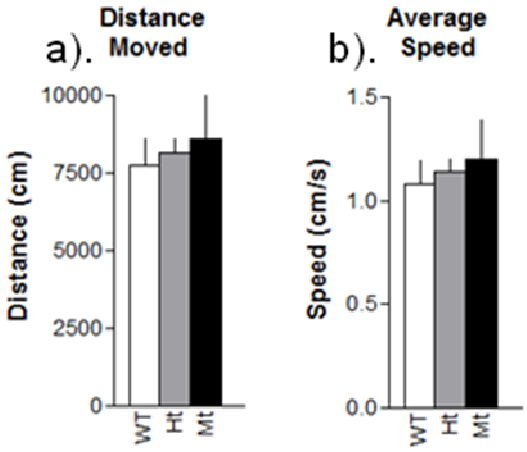


**Figure 2.10:** The effects of αCaMKII autophosphorylation deficiency on homecage activity. Bars represent mean values ± standard error of the mean (SEM).

### 3.5. Experiment 5: Novel Object Exploration

This test examined reactivity to a novel (discrete) stimulus in a familiar arena. Statistical analysis showed a significant difference in exploratory time between genotypes ($F(2, 16) = 4.52$, $p<0.05$; Fig. 2.11a). Post-hoc tests showed a significant decrease in exploratory time in Mt mice compared to WT mice ($p< 0.05$), but not to Ht mice ($p>0.05$). There were no significant differences between the distance (Fig. 2.11b) and average speed (Fig. 2.11c) of movement between groups. These results suggest that the hyperlocomotor response of the αCaMKII mutant mice does not occur towards novel, discrete stimuli in a familiar arena.

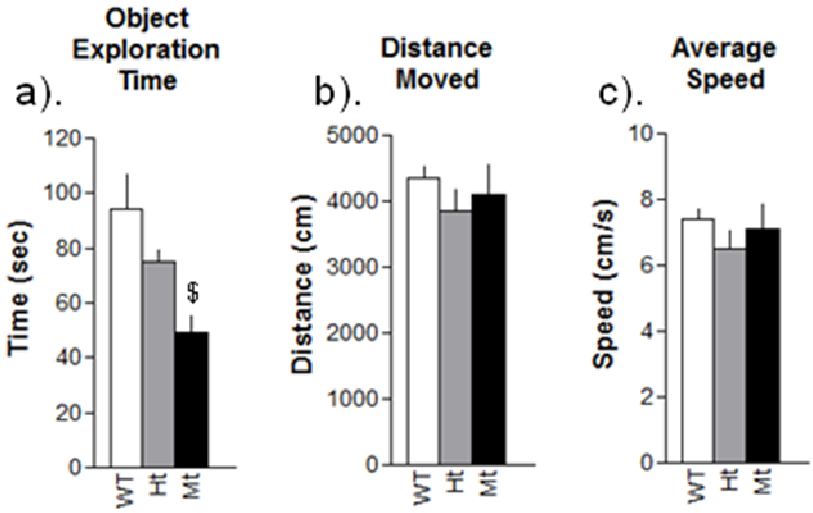


**Figure 2.11:** The effects of αCaMKII autophosphorylation deficiency on novel object exploration. Bars represent mean values ± standard error of the mean (SEM). Post-hoc Tukey HSD test significance: $^{\$}$ $p<0.05$, Tukey HSD test vs. WT.

## 4. Discussion

There is strong evidence for a crucial role of αCaMKII autophosphorylation in learning and memory. Importantly, this role was shown for aversively motivated tasks (Giese *et al.*, 1998; Need and Giese, 2003; Irvine *et al.*, 2005), but not for appetitively motivated tasks (Carvalho *et al.*, 2001). In addition, αCaMKII mutant mice with either a lack of, or knock down or over-expression of αCaMKII show altered emotional behavior in combination with altered locomotor activity (Chen *et al.*, 1994; Lo Iacono and Gross, 2008; Yamasaki *et al.*, 2008; Hasegawa *et al.*, 2009). These findings prompted the question whether αCaMKII autophosphorylation, as a specific function of αCaMKII, may be involved in the control of emotionality and the behavioral response to aversive stimuli.

The present study aimed to explore how αCaMKII autophosphorylation may regulate and control anxiety-like behavioural responses towards threatening stimuli. Open field and light/dark-box tests assessed behaviour of αCaMKII autophosphorylation-deficient mice in novel environments which contain potentially threatening/aversive elements (Rodgers, 1997). Mutant mice were more active in the open field but there was no significant difference in anxiety in the open field. Locomotor results agree with previous reports from mice over-expressing αCaMKII, which showed a significantly reduced locomotor activity in the open field test (Hasegawa *et al.*, 2009). Reduced anxiety-like behaviour in the open field was reported for heterozygous and homozygous αCaMKII deficient mice (Chen *et al.*, 1994) but no differences in the time spent in the centre were observed in the present study. Anxiety-related behaviours were reduced in αCaMKII autophosphorylation-deficient mice in the light/dark test. However, anxiety parameters were confounded by a hyperlocomotor phenotype of the αCaMKII autophosphorylation-deficient mice during testing. These findings are also in line with previous reports on αCaMKII over-expressing mice in the light/dark box. These mice showed an increased latency to enter the light box and a significant reduction in transitions (Hasegawa *et al.*, 2009). In the EPM, αCaMKII autophosphorylation-deficient mice showed significantly less anxiety related behaviour than WT controls and Ht littermates in the EPM. The EPM

distinguished best between anxiety and a hyperlocomotor phenotype. This analysis showed that anxiety was essentially influencing the locomotor activity. Thus αCaMKII autophosphorylation-deficient mice showed an altered response of reduced avoidance to the potentially threatening environment of the open and elevated arms rather than a hyperactive phenotype in this particular test.

Animals may show normal behavioural activity in a familiar and non-threatening environment. In order to test this, mice were monitored in their homecage without any observer interference. Data revealed that αCaMKII autophosphorylation does not play a role in spontaneous locomotor activity under resting conditions, i.e. in a familiar non-threatening environment. This confirmed that αCaMKII autophosphorylation only plays a role in novel situations, where there is a perceived threat. Previous studies which targeted all αCaMKII functions had shown that αCaMKII over-expressing mice showed a significant decrease in home cage activity (Hasegawa *et al.*, 2009), while heterozygous αCaMKII deficiency resulted in an enhanced home cage activity (Yamasaki *et al.*, 2008). Results suggest that while αCaMKII autophosphorylation is not required for spontaneous locomotor activity, the $Ca^{2+}$ dependent function of αCaMKII may, nevertheless, modulate this behaviour in both directions.

In order to test, whether discrete novel stimuli alone would drive hyper-locomotion, the novel object exploration test was performed. This test assessed the behavioural response in a potentially non-threatening environment with a neutral object (Dere *et al.*, 2007). Results showed no differences in locomotor activity between genotypes. αCaMKII autophosphorylation-deficient mice were, however, found to explore the novel object significantly less than WT and Ht littermates. Given that the Mt mice were actively searching and exploring novel environments in the other tests, a general neophobia may not account for the observed effects in this test. These findings further support the view that hyperactivity seen in αCaMKII autophosphorylation-deficient mice under test conditions is not induced by novel stimuli alone. Novel object exploration was reduced in the Mt mice

which may have been a result of the Mt mice actively avoiding the objects or simply due to a reduced exploratory drive.

A threatening stimulus can result in either a flight or fight behavioural response (Blanchard and Blanchard, 1989). Heterozygous αCaMKII deficient mice showed increased defensive aggression (Chen *et al.*, 1994). Mice over-expressing αCaMKII in the forebrain, in contrast, display reduced freezing and fearful behaviours in foot shock tests, but reduced offensive and defensive aggression (Hasegawa *et al.*, 2009). These findings might suggest that αCaMKII facilitates threat-induced behavioural activation and several tests found that mice deficient in αCaMKII autophosphorylation responded to the novel environment with an increased locomotor reactivity.

A potential neuronal mechanism of how αCaMKII autophosphorylation affects behavioural responses to a novel, threatening environment may be found in brain areas which are involved in the processing of aversive stimuli and show a high level of αCaMKII activity. The amygdala is an important brain area when considering emotionality (LeDoux, 1995). It is well known to be associated with the processing of fear and threatening stimuli (Öhman and Soares, 1994; Morris *et al.*, 1998; Carlsson *et al.*, 2004) and it is involved in the processing and formation of emotional memories (Amunts *et al.*, 2005). Damage to the amygdala has been shown to suppress behavioural fear responses to unconditioned and conditioned stimuli (Selden *et al.*, 1991; Phillips and LeDoux, 1992; Armony and LeDoux, 1997). CaMKII is highly expressed in the lateral and basolateral amygdala (Miller and Kennedy, 1986). αCaMKII in particular is present in abundance in the basolateral amygdala (Moriya *et al.*, 2000; Rodrigues *et al.*, 2004), where it is found in pyramidal cells, but not in GABAergic interneurons (McDonald *et al.*, 2002). This makes the basolateral amygdala an interesting candidate region for mediating the influence of αCaMKII autophosphorylation on threat-induced behaviour. Indeed c-fos activation has been found in CaMKII-positive neurons of the basolateral amygdala following restraint stress (Reznikov *et al.*, 2008) or threatening ferret odour stimulation (Butler *et al.*, 2011).

An important projection of the amygdala aims at the periaqueductal gray (PAG) of the midbrain (Armony and LeDoux, 1997). Glutamatergic

receptors and the CaMKII pathway are expressed in PAG neurons (Garzón *et al.*, 2008). The dorsal PAG (dPAG) is one of the key structures in panic disorder, and elicits flight and escape responses and defensive postures (Graeff, 1981; Jenck *et al.*, 1995; Lovick, 2000; de Paula Soares and Zangrossi, 2009). The dPAG has also been suggested to evoke active flight behaviours such as trotting, galloping and jumping (Molchanov and Guimarães, 1999) and to reduce freezing behaviour (Walker *et al.*, 1997). Deep brain stimulation of the dPAG elicits freezing and flight reactions in rats (Moers-Hornikx *et al.*, 2011), exhibited by vigorous running and jumping (Lim *et al.*, 2008). This mimics panic attack in humans (Schenberg *et al.*, 2001; Lim *et al.*, 2008). As threat approaches, brain activity shifts from the forebrain and the amygdala, to the midbrain and the PAG (Maren, 2007).

A possible mechanism of how αCaMKII autophosphorylation controls the amygdala-PAG pathway may be by its effects on AMPA and NMDA receptor function. In an autophosphorylated state, αCaMKII phosphorylates the GluR1 and GluR2/3 subunits of the AMPA receptor and mediates trafficking and insertion of new AMPA receptors into the post synaptic membrane (Barria *et al.*, 1997; Hayashi *et al.*, 1997). This enhances AMPA receptor channel conductance (Derkach *et al.*, 1999; Esteban, 2003). AMPA receptors are present in the dPAG (Albin *et al.*, 1990). Research in cats suggests that the PAG is an important target for excitatory glutamatergic projections from the hypothalamus and amygdala in the modulation of the defensive response to threat (Shaikh *et al.*, 1994; Adamec, 1997). Glutamate injected directly into the PAG has been found to elicit defensive behaviours such as freezing and flight responses (Bandler *et al.*, 1985; Bandler and Carrive, 1988). Ferreira-Netto and colleagues (2005) proposed that glutamate induces defensive behaviours in two different ways. Firstly by activating AMPA receptors, producing a freezing response. Secondly, prolonged presence of glutamate in the cleft will subsequently potentiate NMDA receptors (Ferreira-Netto *et al.*, 2005).

A limitation of this study is the uneven numbers of males and females in each group. This was largely due to availability and additional strict exclusion criteria put in place, designed to eliminate mice which either

escaped from the apparatus, or were completely inactive during the test. However, there was no significant sex effects detected in the sample. Therefore data was pooled between sexes within genotype groups for further analysis (Crusio *et al.*, 2009).

The present study aimed to investigate how αCaMKII autophosphorylation controls behavioural responses towards novel, threatening situations. There was a significant reduction in anxiety-related measures in αCaMKII autophosphorylation-deficient mice throughout various tests of anxiety. However, virtually all anxiety parameters were found to be highly confounded by significantly enhanced locomotor activity. Therefore, the relationship between anxiety-related behaviours and locomotion was investigated. Analysis of covariance indicated that αCaMKII autophosphorylation-deficient mice show an increased locomotor activation induced by novel environments. This response, however, was not seen in a non-threatening environment (home cage) or in response to a discrete, novel stimulus. One might conclude that αCaMKII autophosphorylation does not simply modify anxiety. Instead data suggests that αCaMKII autophosphorylation influences locomotor reactivity to novel environments that are potentially, but not necessarily threatening.

# αCaMKII autophosphorylation mediates the establishment of alcohol reinforcement by altering dopamine-serotonin balance

Chapter 3

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## Abstract

αCaMKII is an important molecule for plasticity of the glutamatergic system in the brain. The autophosphorylation of αCaMKII allows activation of glutamatergic synapses to be potentiated for longer within the cell, thereby accelerating learning. Addiction involves a number of learning and memory processes and αCaMKII is well documented to be important for drug addiction as well as for memory formation. The present study investigated whether αCaMKII autophosphorylation affects the speed at which alcohol-addiction related behaviors were established. Alcohol drinking was initially diminished in αCaMKII autophosphorylation deficient (Mt) mice, but could be established to the same level as WT mice after repeated alcohol withdrawal and reinstatement of alcohol drinking. Alcohol-induced CPP developed at an accelerated rate in Mt compared to WT mice. Taking previous data into account, this may be due to the presence of fast acting negative reinforcement mechanisms in αCaMKII mice. αCaMKII autophosphorylation deficient mice showed no DA response in the NAcc to acute or chronic alcohol administration, but a large enhancement in the 5-HT response in the PFC. The attenuated DA response in mutants correlated with diminished drinking behaviour and altered c-Fos activation in the ventral tegmental area. These data suggest that αCaMKII autophosphorylation contributes towards the establishment of alcohol-addiction related behaviors by altering the DA – 5-HT balance in the meso-corticolimbic system.

## 1. Introduction

Alcohol use can develop into addiction in a significant number of people, and yet the mechanism of transition between alcohol use and dependence is not sufficiently understood. The establishment of addiction remains largely unknown but it is thought to involve a number of distinct learning and memory processes. It is now widely believed that addiction and memory formation share molecular and anatomical pathways (Nestler, 2002a; Kelley, 2004; Hyman, 2005; Müller and Schumann, 2011). In order to investigate the link between these pathways more fully, the present study evaluates whether disruption in a protein which plays a key role in synaptic plasticity can alter measures of alcohol preference, and the rate at which these preferences are established.

αCaMKII plays a key role in the plasticity of glutamatergic post-synapses of the forebrain (Colbran and Brown, 2004; Irvine *et al.*, 2006; Wayman *et al.*, 2008). It is important for memory formation (Elgersma *et al.*, 2004) as well for drug addiction (Anderson *et al.*, 2008). αCaMKII becomes active in the presence of CaM. Upon activation, αCaMKII can switch to an autonomous mode of activity known as autophosphorylation (Giese *et al.*, 1998). This renders the kinase active and free to phosphorylate both neighboring subunits of the holoenzyme at the T286 residue, and its downstream substrates even after the dissociation of the CaM complex. This allows the kinase to remain active for longer which results in signals being potentiated for longer within the cell, thereby accelerating learning. αCaMKII autophosphorylation deficient mice do not show $Ca^{2+}$ independent activation but retain $Ca^{2+}$ dependent activity. If CaMKII is unable to phosphorylate itself, more CaM molecules will be required to activate the entire holoenzyme in order to produce the same effect on learning. In line with this, αCaMKII autophosphorylation deficient mice show severe learning impairments (Irvine *et al.*, 2005), suggesting that autophosphorylation controls the speed of normal learning (Lengyel *et al.*, 2004; Lee *et al.*, 2009). This deficiency does not alter the capacity to learn, long term memory storage or memory retrieval (Irvine *et al.*, 2006).

There is a growing body of research supporting a role for αCaMKII in drug addiction pathways. One of the ways in which alcohol exerts its effects is via the glutamatergic system (Spanagel, 2009), a system in which αCaMKII also plays an important role. Ethanol acts as an NMDA antagonist in dopamine (DA) rich areas of the midbrain including the ventral tegmental area (VTA) and the substantia nigra. These areas have implications for motivation, reward and addiction (Yang *et al.*, 1996). Alcohol administration also enhances DA release from the mesolimbic pathway (Di Chiara and Imperato, 1988) with preferential release from the nucleus accumbens (NAcc) (Pontieri *et al.*, 1995). In addition, alcohol increases αCaMKII expression levels in rat cerebral cortex which can be attributed to $Ca^{2+}$ influx during chronic alcohol exposure (Mahadev *et al.*, 2001).

Given the reported differences in learning and memory tasks and the potential interaction with drugs of abuse, αCaMKII autophosphorylation may play a role in the motivational and rewarding effects of alcohol. Consistent with previous findings the main hypothesis of this study states that αCaMKII autophosphorylation may control the speed at which alcohol-addiction related behaviors are established, but does not play a role in the processes responsible for the maintenance of addiction once established. In order to evaluate the establishment of alcohol preference, chronic alcohol drinking will be monitored in a two-bottle free-choice drinking paradigm, as well as alcohol-induced conditioned place preference. Systemic alcohol measures will also be taken to rule out any confounding differences in the metabolism of alcohol. The acute positive reinforcing effects of alcohol are mediated by the recruitment of specific neurotransmitter systems in the reward centres of the brain (Koob *et al.*, 1998a; McBride, 2010). In vivo microdialysis performed in brain regions associated with drug reward will therefore examine monoaminergic responses which might contribute to the incentive properties, emotional effects and potential arousal regulating effects of alcohol. Regions to be dialysed have been selected based on the degree of overlap between the regions in which DA and other monoamines are released in response to alcohol administration, and regions in which αCaMKII is abundantly

expressed. Alcohol administration induces DA release from the mesolimbic pathway, and the NAcc in particular (Pontieri *et al.*, 1995; Di Chiara and Imperato, 1988). The αCaMKII isoform is most prominently expressed in the forebrain at glutamatergic post-synapses (Lisman *et al.*, 2002; Colbran and Brown, 2004; Irvine *et al.*, 2006; Wayman *et al.*, 2008). For this reason, the NAcc was selected as the first target region. The prefrontal cortex (PFC) was nominated as the second target region. This selection was based on the previously mentioned criteria, and because the PFC receives input from projections of the NAcc. DA and 5-HT mediate the motivational and emotional effects of alcohol (Spanagel, 2009; McBride, 2010). To further investigate the mechanisms which may influence dopaminergic activation, neuronal activity will be measured in the VTA. c-Fos activation will be measured in the VTA since the VTA has projections directed to regions of the brain associated with drug reward.

## 2. Materials and Methods:

All housing and experimental procedures were performed in accordance with the U.K. Home Office Animals (Experimental Procedures) Act 1986.

### 2.1 Animals

Male and female αCaMKII$^{T286A}$ mice (Giese *et al.*, 1998) were studied in sex balanced designs in all experiments (for details of the genetic background: see Chapter 2, Materials and Methods; section 2.1). This mutation blocks the autophosphorylation of CaMKII but does not affect the $Ca^{2+}$ dependent activity (Giese *et al.*, 1998). Animals were individually housed, provided with food and water ad libitum, and kept on a 12:12 hour light: dark cycle (lights on at 7.00 am). Behavioral tests were performed during the light cycle between 09:00 and 16:00 h. Room temperature was maintained between 19℃ and 22℃ at a humidity of 55% (±10%). Animals were housed in Tecniplast cages (32cm x 16cm x 14cm), using Litaspen sawdust and nesting materials, (Sizzlenest, Datsand, Manchester UK).

### 2.2 Alcohol drinking and deprivation effect

Alcohol drinking was tested in naïve Mt (n=10), wild-type (WT; n=12) and heterozygous (Ht; n=12) animals using a two-bottle free-choice drinking paradigm. Each cage was equipped with two continuously available bottles, one of which contained tap water the other bottle contained alcohol in various concentrations. After an acclimatization period to establish a drinking baseline to the water in both bottles, animals received alcohol at increasing concentrations of 2, 4, 8, and 12 vol. %. Mice were exposed to each concentration of alcohol for 4 days before exposure to a higher concentration of alcohol. Thereafter, alcohol concentration was switched to 16 vol. % and animals were allowed to drink for 2 weeks. In order to measure the alcohol deprivation effect (Spanagel and Hölter, 2000), baseline consumption of 16 vol % alcohol was measured. Alcohol was then removed for 3 weeks (both bottles contained tap water) before it was re-introduced for 4 days. This procedure was repeated once more. Bottles were changed and weighed daily.

The consumed amount of alcohol relative to body weight and the preference versus water were measured. Control cages holding only water and alcohol bottles were arranged alongside test cages. Spillage was measured and all data were corrected for bottle spillage prior to analysis.

### 2.3 Taste preference test

Alcohol experienced animals were used for this test (Mt: n=10; WT: n=12; Ht: n=12). Sucrose (0.5 and 5%) and quinine (10 and 20 mg/dl) preference was measured in a two-bottle free-choice test vs. water. Each dose was offered for 3 days with the position of the bottles being changed and weighed daily (Spanagel *et al.*, 2005a).

### 2.4 Determination of blood alcohol levels

Alcohol naïve animals were used for this test (Mt: n=11; WT: n=12; Ht: n=14). Animals were injected intraperitoneally (i.p.) with alcohol (3.5g/kg). Mice were left undisturbed and then systematically culled at 15, 30 and 60 minutes post injection. Animals were immediately sacrificed by cervical dislocation and trunk blood was collected. Blood was left at room temperature for 30-60 min to allow clotting. The blood was then placed in a centrifuge for 10-15 mins at 3000-4000 rpm at room temperature. The supernatant was quickly aspirated taking care not to disturb the cell layer. Serum samples were stored at -80℃ waiting further analysis. Analysis of the alcohol concentration in the serum samples was performed using a quantitative enzymatic method. Briefly, after predilution of the samples to obtain an appropriate volume, the samples were assayed with Integra 400 Plus (Roche) analyzer. In the presence of nicotinamide adenine dinucleotide (NAD+), alcohol dehydrogenase oxidizes ethanol to acetaldehyde. Simultaneously generated NADH, which is directly proportional to the ethanol concentration in the sample, is measured photometrically. In each analytical run, appropriate quality control samples were analyzed.

## 2.5 Conditioned place preference (CPP)

The Conditioned Place Preference paradigm is a form of Pavlovian conditioning which is used to examine the rewarding (CPP) or aversively (Conditioned Place Aversion; CPA) motivating effects of stimuli (toys or food) or experiences (drugs of abuse) (Cunningham *et al*., 2006). In the following experiments, the CPP design was used to examine the rewarding effects of alcohol. Mice were essentially allowed to make a choice between a drug associated cue and a neutral cue, and then the motivational significance of the preferred compartment was assessed. The method used was a modified version of Cunningham's experimental design (Cunningham *et al*., 2006). This included repeated preference testing systematically placed between CPP conditioning and extinction trials in order to determine a learning curve for CPP, according to the delayed establishment hypothesis.

While CPP cannot model addiction as a disease state, the paradigm can be used to assess aspects of addiction and related behaviours by looking at the motivational properties of various drugs and is useful for studying the effects of environmental cues associated with drug experiences (Bardo and Bevins, 2000). There are several advantages of using the CPP test design, including; simple methodology which does not require complex surgical procedures, the potential for high throughput, the ability to measure both appetitive and aversive effects in the same procedure, its sensitivity to low-dose effects and the facility of measuring drug induced changes simultaneously to locomotor activation (Cunningham *et al*., 2006). There is some difficulty when using the CPP test design to study alcohol reward. Methodological parameters can greatly influence the outcome of these experiments (Tzschentke, 1998). Outcome may be dependent upon route of administration and the dosage used (van der Kooy *et al*., 1983; Asin *et al*., 1985), and the relative timing of ethanol administration and subsequent exposure to the conditioned stimulus. For example, ethanol administration prior to exposure to the conditioned stimulus leads to CPP, and administration following exposure to the conditioned stimulus causes CPA to develop (Cunningham, *et al*., 1997). The precise timing of administration prior to testing (5, 10, 15 or 30 minutes) has also been shown to alter the

development of CPP (Cunningham, *et al*., 1997). The sensitivity of this procedure to ethanol's rewarding effect appears to differ considerably between species (Cunningham et al., 1993). Differences have also been noted within species using different inbred and out-bred strains (Risinger and Oakes, 1996; Cunningham and Noble, 1992; Gevaerd and Takahashi, 1996), and even between selectively bred high and low sensitivity mouse strains Risinger *et al*., 1994) and in alcohol-preferring and non-preferring rat lines (Schechter, 1992). The CPP paradigm has been criticised as being less relevant for the study of drug reinforcement when compared to procedures in which animals self-administer drugs (Dworkin and Smith, 1988). However, contemporary approaches recognise the importance of Pavlovian learning processes in the development of drug addiction (Robinson and Berridge, 1993; Everitt and Robbins, 2005).

*2.5.1 Apparatus*

The TSE Place Preference test boxes (Bad Homburg, Germany; Fig. 3.1) were made of non transparent PVC with standard inside dimensions of 40cm (L) x 15cm (W) x 20cm (H). The apparatus was divided into 3 fully automated compartments, the outer chambers measured 17cm in length, and the centre chamber 6cm. The floor of the left chamber (compartment A) was covered with a smooth black rubber mat. The floor of the right chamber (compartment B) was covered with a patterned black rubber mat. The centre chamber was not covered and coloured white (compartment C). Activity was recorded in each compartment using high-resolution infrared sensors. The system automatically recorded the number of entries made and distance moved in each compartment for each trial. An unbiased design was used, i.e. half the mice were conditioned to their preferred compartment, and half to their non-preferred compartment.

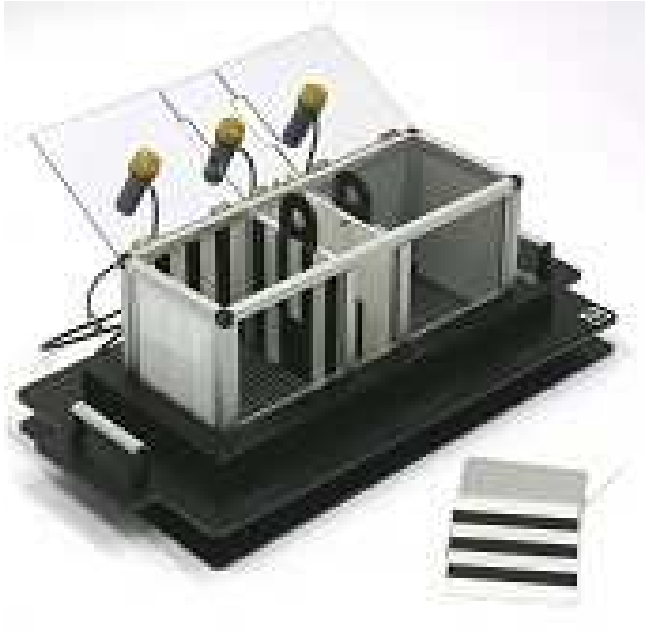


**Figure 3.1:** Conditioned Place Preference Apparatus (Bad Homburg, Germany)

2.5.2 *CPP Establishment*

Alcohol naïve animals (Mt: n=11; WT: n=12; Ht: n=14) were injected (i.p.) immediately before each trial with either saline, or ethanol solution at a dose of 2g/kg. Mice were instantly transferred to the testing suite and placed into the CPP boxes, signifying the beginning of the trial period (Fig. 3.2). The experiment involved four phases (Table 3.1); habituation trial (one session), conditioning trials (14 sessions), preference tests (5 sessions) and an incubation period (7 days). Trials were performed once daily. *Habituation (day 1):* The habituation session was intended to acclimatise mice to the test procedure and apparatus prior to commencing the experiment. Mice were injected with saline and introduced into the centre compartment with free access to all three compartments for 20 minutes. *Pre-test (day 2):* The pre-test was designed to establish a baseline level of preference for each individual animal. Mice were conditioned to either their preferred or non preferred compartment using a counterbalanced experimental design. Mice were injected with saline and introduced into the centre compartment with free access to all three compartments for 20 minutes. *Conditioning trials (days 3-4, 6-9 and 11-18):* Conditioning trials were performed in pairs, odd numbered

pairings were conditioned with alcohol, and even numbered pairings were conditioned with saline, this was balanced across groups. All animals received 7 pairings with saline and 7 pairings with alcohol. Mice were injected with either saline or an ethanol solution (2g/kg i.p.) and introduced into one of two compartments, with restricted access, for 5 minutes (Cunningham *et al.*, 2006). *Preference tests (days 5, 10, 19 and 27):* In order to monitor the time course of CPP establishment, preference tests were systematically dispersed between conditioning trials on days 5, 10, 19 and 27. Before each test, mice were injected with saline and introduced into the centre compartment with free access to all three compartments for 20 minutes. *Incubation period (days 20-26):* In order to test the consolidation of CPP, animals were left undisturbed in their home cages on days 20-26, and then re-tested for CPP.

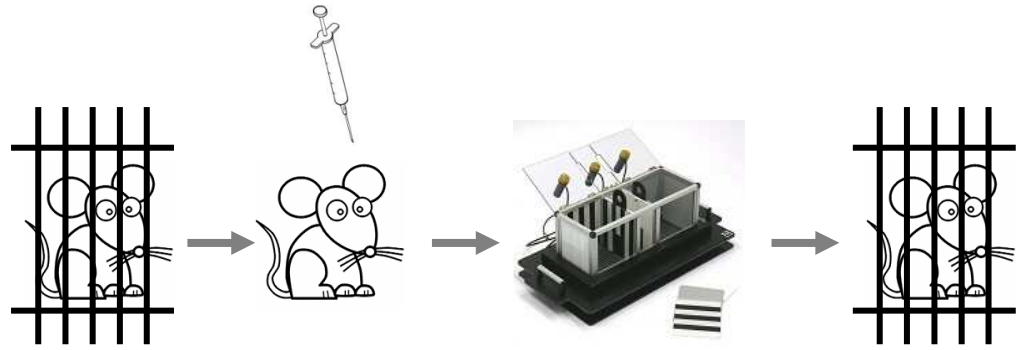



**Figure 3.2:** Conditioned Place Preference procedure, illustrating the test procedure. Each mouse was removed from the homecage and injected with alcohol/saline solution. The mouse was then immediately transferred to the testing suite and placed in the CPP test box. When the test was complete, mice were returned to their homecage.

**Table 3.1:** The entire experimental protocol each mouse was exposed to during the conditioned place preference procedure

| Trial | Duration | Treatment |
|---|---|---|
| Habituation | 5 mins | Saline |
| Pre Test | 20 mins | Saline |
| Conditioning x 2 | 5 mins | Alcohol/Saline |
| Preference Test 1 | 20 mins | Saline |
| Conditioning x 4 | 5 mins | Alcohol/Saline |
| Preference Test 2 | 20 mins | Saline |
| Conditioning x 8 | 5 mins | Alcohol/Saline |
| Preference Test 3 | 20 mins | Saline |
| Incubation | 7 days | None |
| Preference Test 4 | 20 mins | Saline |

*2.5.3 CPP Extinction*

*Extinction Trials (days 28, 30-31, 33-36 and 38-41):* Extinction trials were designed to intentionally pair the previously alcohol-paired compartment with saline, in an attempt to extinguish any learnt preference. Two sessions were performed each day, one in each compartment. Mice were injected with saline before each trial and introduced into one of two compartments, with restricted access, for 5 minutes. *Reconditioning trials (days 43-44):* Reconditioning trials were performed in pairs, to mirror the conditioning trial procedure, to determine whether an extinguished, learnt behaviour could be reinstated using a single alcohol-paired conditioning trial. Mice were injected with either saline or an ethanol solution (2g/kg i.p.) and introduced into one of two compartments, with restricted access, for 5 minutes. One session was performed on each test day. *Preference tests (days 29, 32, 37, 42 and 45):* Preference tests were systematically placed between extinction trials to assess the time course of CPP extinction (Table 3.2). Before each test, mice were injected with saline and introduced into the centre compartment with free access to all three compartments for 20 minutes (refer to Fig. 3.2).

**Table 3.2:** The entire experimental protocol each mouse was exposed to during the conditioned place extinction and reinstatement procedure

| Trial | Duration | Treatment |
|---|---|---|
| Extinction x 2 | 5 mins | Saline |
| Ext Pref Test 1 | 20 mins | Saline |
| Extinction x 4 | 5 mins | Saline |
| Ext Pref Test 2 | 20 mins | Saline |
| Extinction x 8 | 5 mins | Saline |
| Ext Pref Test 3 | 20 mins | Saline |
| Extinction x 8 | 5 mins | Saline |
| Ext Pref Test 4 | 20 mins | Saline |
| Re-Conditioning x 2 | 5 mins | Alcohol/Saline |
| Re-Cond Pref Test | 20 mins | Saline |

### 2.6 Loss of righting reflex (LORR)

Alcohol naïve animals were used for this test (Mt: n=5; WT: n=5; Ht: n=5). Animals were administered with an ethanol injection of 3.5g/kg (i.p.) (Spanagel *et al.*, 2002) to induce LORR, and immediately placed in a clean and empty cage. LORR was observed when the animal became ataxic and stopped moving for at least 30 seconds. The animal was then placed on its back. Recovery from alcohol administration was defined as the animal being able to right itself three times within a minute. Some animals took a long time to recover, so a 2 hour cut off was used. The time taken for the animal to lose its righting reflex, and the time to recovery from alcohol's effect on righting reflex, were observed and recorded. LORR was carried out on day one of the experiment when animals received the first of 8 alcohol injections (3.5g/kg, i.p.). Upon completion of the trial, the animal was returned to the home cage. Then, alcohol was administered once daily on days 2-7, i.e. between LORR experiments. Animals were weighed and injected 2g/kg (i.p.). LORR was

again tested on day eight of the experiment when animals received the last of 8 alcohol injections (3.5g/kg, i.p.).

## 2.7 In-vivo Microdialysis

Reduced alcohol drinking behaviour seen in αCaMKII Mt mice suggested that the acute rewarding properties of ethanol may also be reduced. The DA system is critical to experience the acute reinforcing effects of alcohol (Koob, 1992; McBride and Li, 1998; Nestler, 2005), with alcohol typically inducing around a 20% increase in DA levels in the NAcc (Doyon *et al.*, 2003). In order to test whether the lack of αCaMKII autophosphorylation would lead to altered monoaminergic responses to acute or sub-chronic alcohol treatment, in vivo microdialysis was performed after acute and again after sub-chronic alcohol treatment in the same animals. This design allowed a direct comparison of acute and sub-chronic responses in the same animals. The aim was to investigate whether well defined dopaminergic, serotonergic and noradrenergic responses were altered. Acute alcohol challenge alters glutamate levels in the NAcc and affects the function of the reward system (Szumlinski *et al*., 2005). However, measuring extracellular glutamate levels require a different HPLC set-up with UV detection and cannot be measured alongside the other transmitters of interest. Not having access to an HPLC system capable of doing so meant that priority was given to the measure of extracellular DA, 5-HT and NA. Due to the high technical demand a strongly focused design was used, according to Spanagel *et al.,* (2005b) and Szumlinski *et al*., (2005).

### *2.7.1 Surgery*

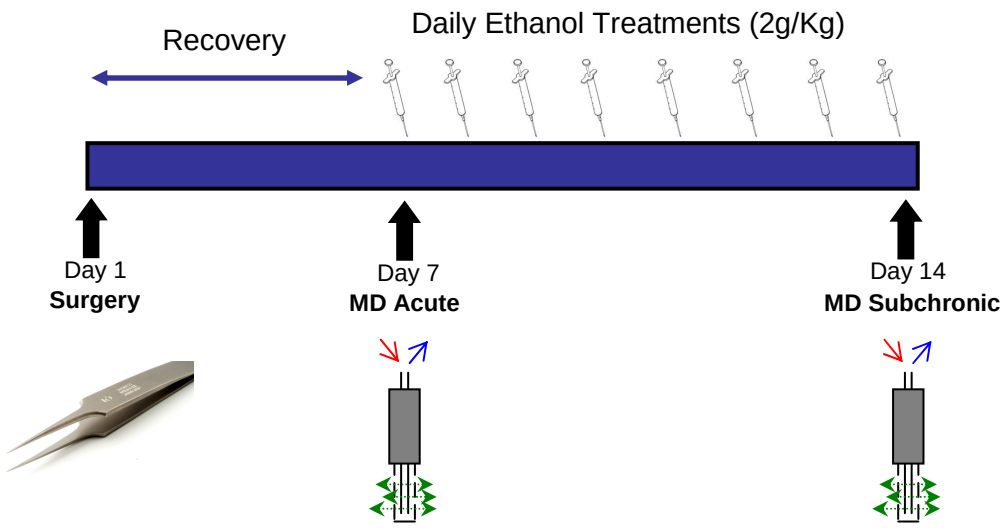
The mice were deeply anaesthetised using a mixture of 4.12ml saline (NaCl), 0.38ml Ketaset (containing 100mg/ml Ketamine) and 0.5ml Domitor (containing 1mg/ml Medetomidine hydrochloride) administered interaperitoneally (i.p.) at 0.1ml per 10g body weight. In addition 0.01ml Rimadyl (5mg/kg Carprofen) analgesia was given subcutaneously (s.c.). The animal was placed in a Kopf stereotaxic frame. Two guide cannulas

(Microbiotech/se AB, Stockholm, Sweden) were aimed at the PFC (A: +1.9; L: ±0.8; V: -1.3 angle ±10º from midline) and the NAcc (A: +1.2; L: ±1.6; V: -4.3 angle ±10º from midline) using coordinates relative to bregma (Franklin and Paxinos, 1997), and fixed in place using two anchor screws (stainless steel, d=1.4mm) and dental cement. Anaesthesia was reversed by administering a mixture of 3.9ml saline (NaCl) and 0.1ml Antisedan (containing 5mg/ml Atipamezole) at 0.08ml/ 10g body weight (s.c.) after approximately 45 minutes. Animals were kept warm and allowed to recover from the anaesthetic. Animals were then returned to their home cages and monitored daily, allowing at least 5 days for complete recovery (Fig. 3.3A).

*2.7.2 Procedure*

On the day of the experiment, microdialysis probes of a concentric design (Fig. 3.3B), membrane lengths were 2mm for the PFC (MAB 6.14.2.) and 1mm (MAB 6.14.1.) for the NAcc, were inserted into the guide cannulae under a short (3-5min) Isoflurane anaesthesia ($O_2$ at 1L/min, Isoflurane at 3% to induce and 2% to sustain). After probe insertion, the animal was placed into an open field (21x21x30cm) of a Truscan system (Coulbourn Instruments, Allentown, USA). Food and water were given ad libitum and room temperature maintained between 19- 22 ºC. The microdialysis probes were connected to a microinfusion pump (CMA 400, Carnegie, Sweden) via a swivel mounted on a balanced arm above the chamber, and were perfused with artificial cerebrospinal fluid (aCSF) (containing $Na^+$ 147 mmol, $K^+$ 4 mmol, $Ca^{2+}$ 2.2 mmol, $Cl^-$ 156 mmol, pH = 7.4) at room temperature (Müller *et al.*, 2007). The flow rate was set to 1.5µl/min and allowed to stabilise for at least two hours until a stable baseline was obtained. Samples were collected every 20 minutes into vials containing 2.73µl of antioxidant (0.1 M perchloric acid and 500 pg dihydroxybenzylamine (DHBA) as internal standard). Three samples were taken during the first testing hour of the experiment to measure baseline quantities of the neurotransmitters DA, 5-HT and NA (Pum *et al.*, 2008b). An injection of alcohol was then administered i.p. (2g/kg, $v_{inj}$= 10 ml/kg). A further nine samples were collected simultaneously to behavioural data collection.

**A).**




**B).**

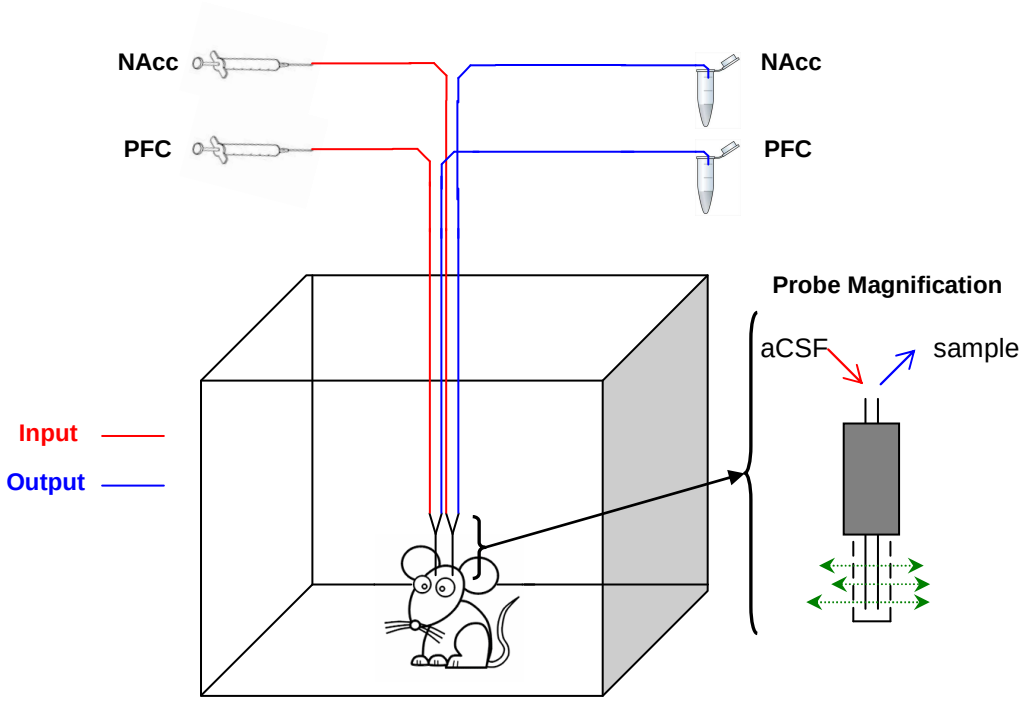



**Figure 3.3:** Microdialysis procedure set-up. **A).** In vivo microdialysis experimental design, reflecting test procedure for each mouse. **B).** Representation of microdialysis set-up and magnification of probe membrane, inserted into the NAcc and PFC and perfused with artificial cerebrospinal fluid (aCSF).

*2.7.3 Acute and subchronic alcohol effects*

Alcohol naïve animals were used for this test (Mt: n=12; WT: n=11; Ht: n=12). In-vivo microdialysis was carried out on day one of the experiment when animals received the first of 8 alcohol injections (2g/kg, i.p.). This provided an acute neurochemical and behavioural response to alcohol treatment. After the microdialysis trial was completed the animal was anaesthetised using an oxygen and Isoflurane mix ($O_2$ at 1L/min, Isoflurane at 3%). The probes were removed and guides re-inserted. The animal was then returned to the home cage. Alcohol was then administered once daily on days 2-7 i.e. between in vivo microdialysis trials (2g/kg, i.p.). In vivo microdialysis was carried out again on day eight of the experiment providing a subchronic neurochemical and behavioural response to alcohol treatment. Once microdialysis experiments were complete, animals were sacrificed by cervical dislocation. Brains were fixed in 4% formaldehyde solution and stored at 4 ℃. Brains were sliced on a microtome and stained with cresyl violet for analysis of probe placement (Fig. 3.4 & 3.5). Since all probes were surgically inserted accurately within the PFC and the NAcc regions, all animals were considered for data analysis.

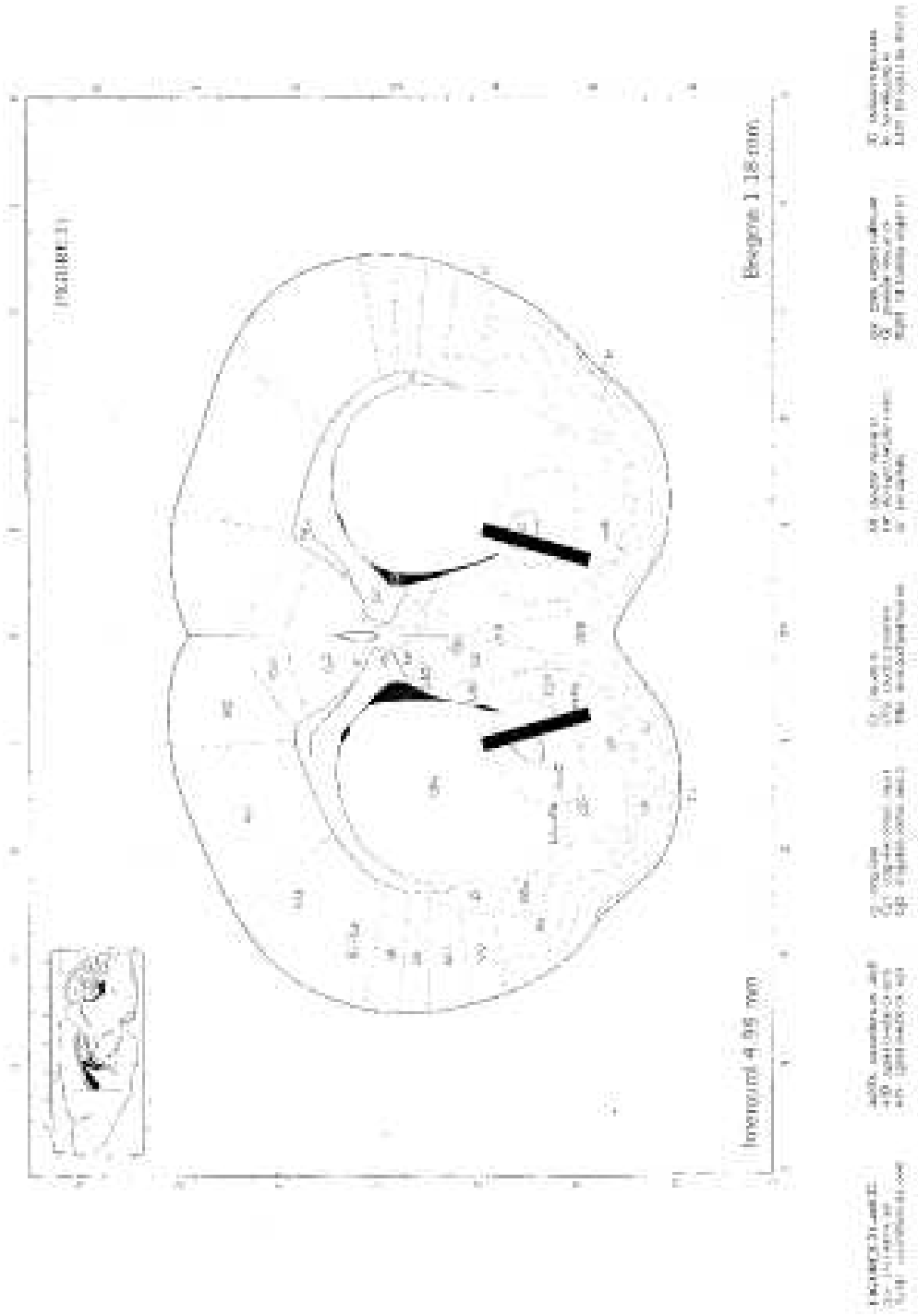



**Figure 3.4:** Lines represent target coordinates for active membrane surface of microdialysis probe into the NAcc. Taken from Franklin and Paxinos (1997) mouse brain atlas.

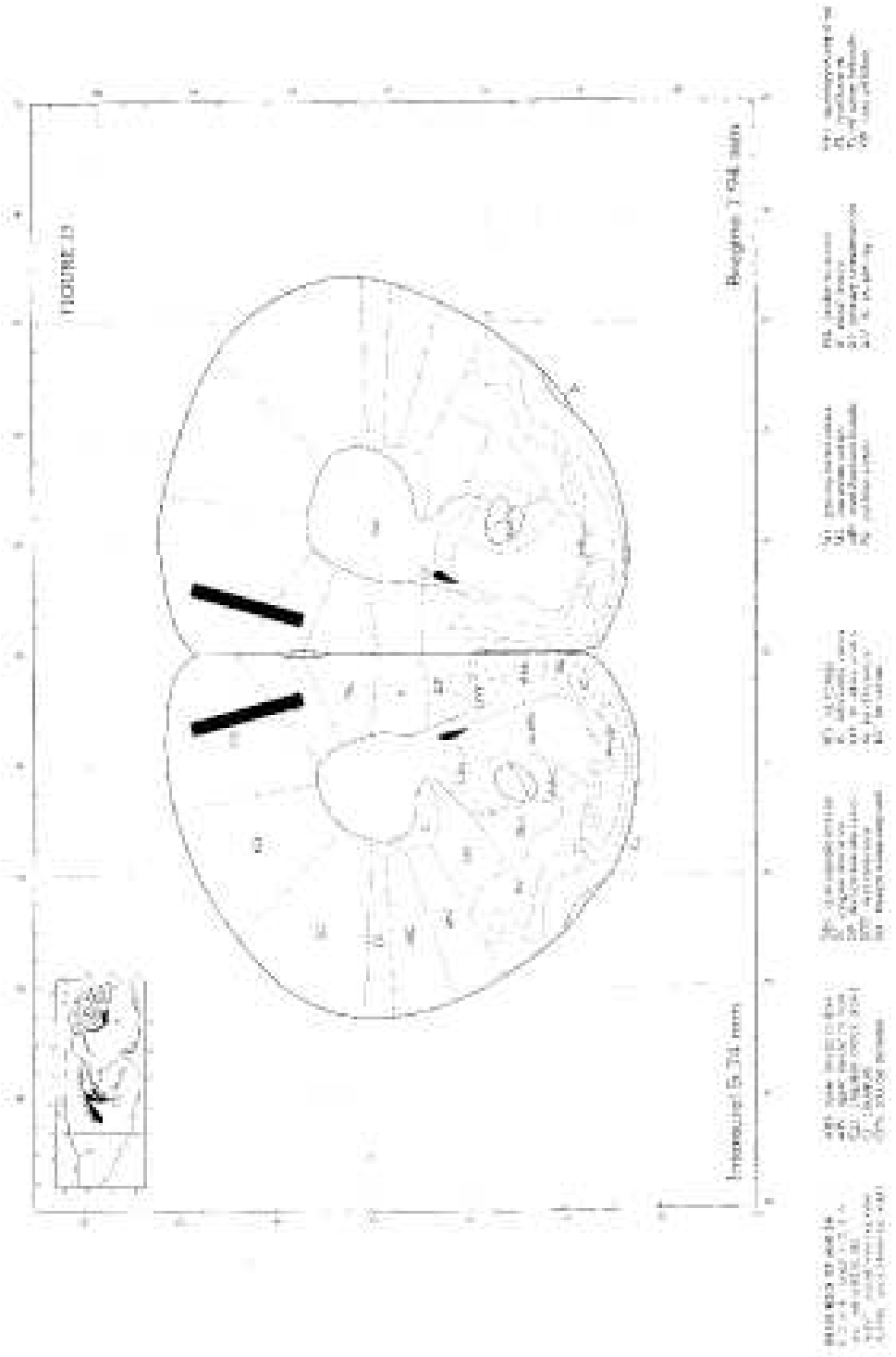
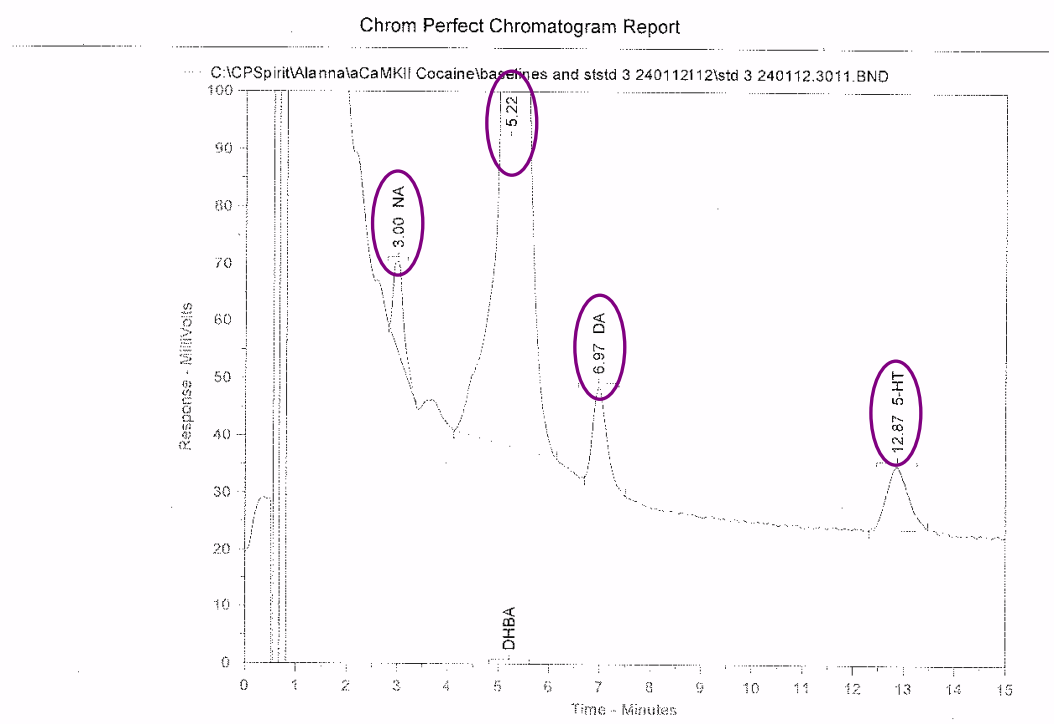


**Figure 3.5:** Lines represent target coordinates for active membrane surface of microdialysis probe into the PFC. Taken from Franklin and Paxinos (1997) mouse brain atlas.

*2.7.4 High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ED) analysis*

All samples were analysed using HPLC-ED to measure DA, 5-HT and NA levels (see Fig. 3.6 & 3.7). The column was an ET 125/2, Nucleosil 120-5, C-18 reversed phase column (Macherey–Nagel, Germany) perfused with a mobile phase composed of 75 mM NaH2PO4, 4 mM KCl, 20µM ethylenediamine tetraacetic acid (EDTA), 1.5 mM sodium dodecyl sulfate, 100 µl/l diethylamine, 12% methanol, and 12% acetonitrile adjusted to pH 6.0 using phosphoric acid. The electrochemical detector (Intro, Antec, The Netherlands) was set at 500 mV vs. an in situ Ag/AgCl (ISAAC) reference electrode (Antec, Leyden, Netherlands) at 30℃. This setup allows the simultaneous measurement of NA, DA and 5-HT. The concentration of many neurotransmitters in the extracellular space is quite low (in the nM – pM range). As a result, the dialysate collected is only a reflection (a few percent) of the true extracellular levels (Sarre and Michotte, 2007). The detection limit of the assay was 0.1pg for all neurotransmitters with a signal–noise ratio of 2:1. Neurochemical data were not corrected for recovery (Pum *et al.*, 2007; 2008b).




Sample Name = **std 3 240112**

Instrument = Instrument 1
Heading 1 =
Heading 2 =

Raw File Name = **C:\CPSpirit\Alanna\std 3 240112.3011.RAW**
Method File Name = C:\CPSpirit\Methods\Test I.MET
Calibration File Name = C:\CPSpirit\calib CPM I.CAL

Date Taken (end) = 24/01/2012 09:05:41
Method Version = 21
Calibration Version = 4179

| Peak # | Ret. Time | Name | Amount | Amt % | Area | Area % | Type | Width |
|---|---|---|---|---|---|---|---|---|
| 1 | 3.00 | NA | 30.41 | 34.632 | 222917 | 25.546 | BB | 0.31 |
| 2 | 5.22 | DHBA | 1.00 | 1.139 | 11780220 | 1350.026 | BB | 0.28 |
| 3 | 6.97 | DA | 28.37 | 32.306 | 316465 | 36.267 | BB | 0.32 |
| 4 | 12.87 | 5-HT | 29.03 | 33.062 | 333211 | 38.186 | BB | 0.48 |

Total Area = 872592.4 Total Height = 43630.34 Total Amount = 87.81194

**Figure 3.6**: Chromatogram report. Example of an HPLC-ED chromatogram of a standard solution containing known amounts of DA, 5-HT, NA and the internal standard DHBA, used for calibration. Peaks are integrated and the area under the peak is calculated. This area is compared to that of DHBA and used to generate precise quantities of DA, 5-HT and NA in picograms.

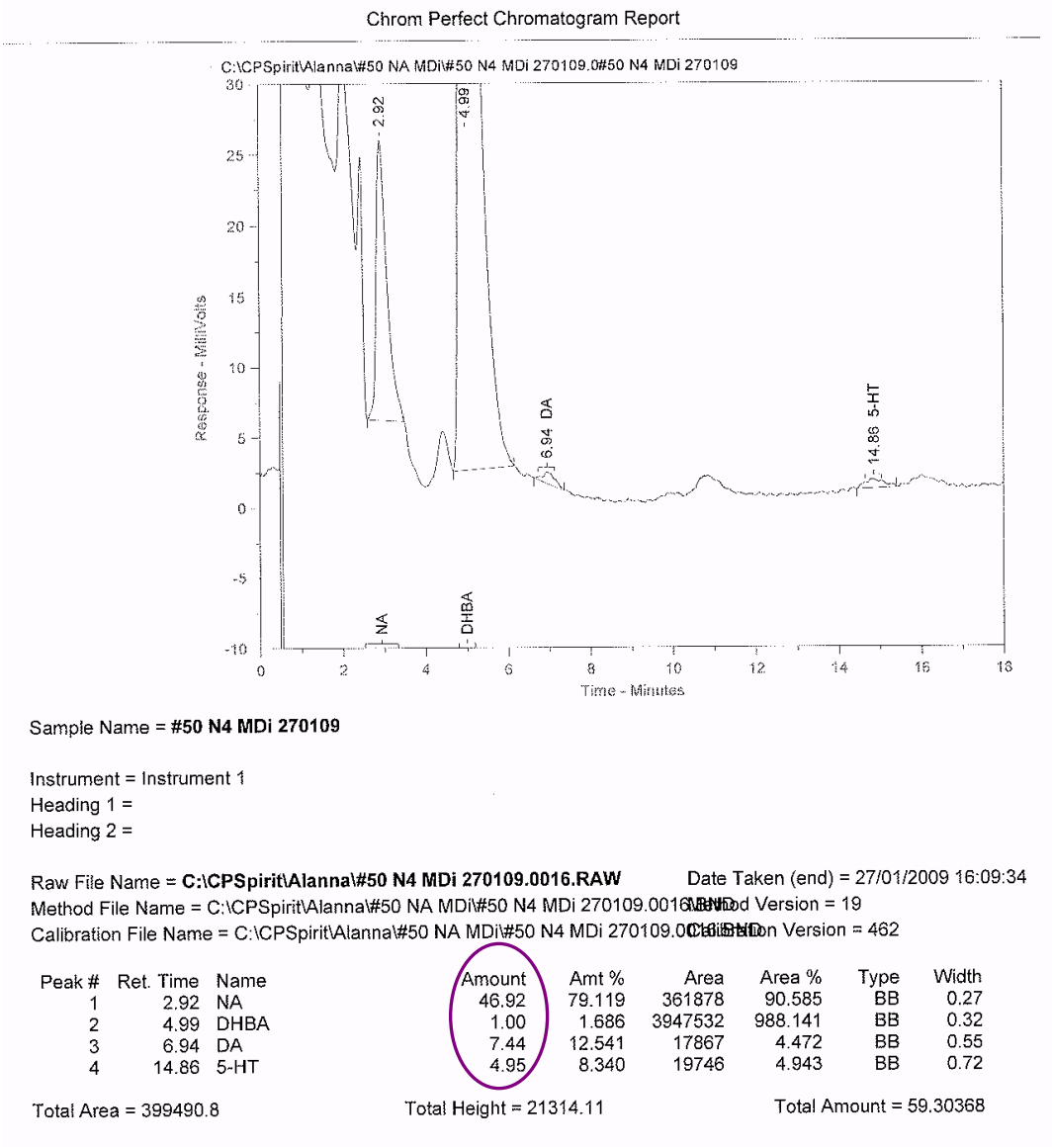



**Figure 3.7:** Real chromatogram report of a sample trace. Trace is from a WT mouse, taken from the NAcc during an acute microdialysis trial, immediately after alcohol injection. Integrated transmitter peak area is compared to that of DHBA and the amount of DA, 5-HT and NA is calculated in picograms.

*2.7.5 Behavioural analysis*

Locomotor activity was measured concurrent with the in-vivo microdialysis experiments. Locomotor activity was automatically measured by a TruScan system (Coulbourn Instruments, Allentown, USA).

## 2.8 c-Fos activation after acute and subchronic alcohol treatment

*2.8.1 Procedure*

Alcohol naïve animals were used for this test (Mt: n=12; WT: n=12). Animals were transferred from the homecage, to a temporary cage and injected either once (acute) or on seven consecutive days (subchronic) with ethanol at a dose of 2g/kg (i.p.). Mice were left undisturbed for 70 minutes after injection minimising further stimuli. After 70 minutes mice were culled under isoflurane narcosis and transcardially perfused with 0.1M PBS for 10 minutes and then fixed with 4% paraformaldehyde (PFA) solution for a further 10 min (flow rate 4ml/min). The brain was removed and left in 4% PFA solution overnight at 4℃. Brains were then transferred to a 30% saccharo se solution and stored at 4℃ until the brains were fully submerged. Brains were then snap frozen in isopentane at -60℃ and stored at -80℃ until the w hole brain was cut into 40 µm coronal sections by cryosectioning. All sections were collected and then stored at -20℃ in an anti-freezing solution until processed for immunohistochemical staining. The floating coronal sections were incubated with an anti-c-Fos rabbit polyclonal antibody (1:30.000, Calbiochem, Germany) for 20 hours. c-Fos immunoreactive cells were visualized using a biotinylated donkey anti-rabbit secondary antibody (1:500, Santa Cruz, Germany) and the avidin-biotin complex (ABC-Elite kit rabbit, Vector Laboratories, Germany) (Richter *et al.*, 2005).

*2.8.2 Stereological quantification*

The number of c-Fos-immunoreactive cells in the rostral and caudal VTA was determined using stereological quantification. The examined regions were defined according to the stereotaxic coordinates (Franklin and Paxinos, 1997).

Stereological quantification of the c-Fos positive cells was carried out strictly blind to the experimental conditions with the optical fractionator estimating total numbers of c-Fos positive cells (Gundersen, 1986; Schmitz *et al.*, 2000; von Horsten *et al.*, 2000). After histological processing the sections had a mounted section thickness of 30 µm, a fixed distance of 2 µm and an optical dissector height of 26 µm, measured with an electronic microtator attached to the microscope. All counting procedures and measurements of reference volumes were conducted on a light microscope (Nikon Eclipse 80i) equipped with a semiautomatic stereology system (Stereoinvestigator, Version 8.27, MicroBrightField, Colchester, Vermont, USA). C-Fos-positive cells were counted within a 70x70 µm counting frame, which was spaced in a 90x90 µm counting grid. Positive cells were counted, if their nucleus came into focus. Positive cells, which intersected the uppermost focal plane (exclusion plane) or the lateral exclusion boundaries of the counting frame were not counted. The total counts of positive c-Fos cells were multiplied by the ratio of reference volume to sampling volume in order to obtain the estimated number of c-Fos-positive cells for each structure.

### 2.9 Statistical Analysis

All graphical output data is expressed as a mean ± SEM. *Alcohol drinking:* Drinking and taste preference data were analysed using two-way ANOVA followed by planned pairwise comparisons using Fisher's LSD with Bonferroni correction. *Microdialysis:* Baseline neurochemical and behavioural data were analysed using one way ANOVA and planned pairwise Fisher's LSD tests. Planned pair-wise comparisons (independent by variable t-tests) were performed to determine differences between acute and subchronic time points within each genotype group. Alcohol induced neurochemical effects were expressed as a percentage of the mean of the three baseline samples which were taken as 100%. Data were compared using two-way ANOVA for the factors genotype (3) and time (12). To compare alcohol effects at certain time points, pairwise comparisons were performed using Fisher's LSD tests. Planned t-test pairwise comparisons were performed to determine differences

between acute and subchronic time points within each genotype group. *LORR:* Data were analysed using two-way ANOVA for the factors genotype (3) and time (2). To compare alcohol effects across time, within genotype groups, planned pairwise comparisons were performed using Fisher's LSD tests. *Blood alcohol levels:* Data were analysed using two-way ANOVA for the factors genotype (3) and time (3). To compare alcohol effects across time and within genotype groups, pairwise comparisons were performed using Fisher's LSD tests. *CPP:* all data were analysed using two-way ANOVA followed by planned pairwise Fisher's LSD tests. *c-Fos expression:* all data were analyzed by one-way ANOVA followed by t-tests. Although sex differences are well known in alcoholism-related behaviors (Desrivières *et al.*, 2011; Lenz *et al.*, 2011), there were no significant sex differences in this study. Therefore, data were collapsed for analysis. The software SPSS 17.0 and Statistica 9 were used. A significance level of $p < 0.05$ was used to test for statistical significance.

As discussed in Chapter 2, Section 2.3. Statistical Analysis, the present study retrospectively used the 'Resource Equation' (Mead, 1988) method to quantify the probability that experiments detected biologically important effects. Mead's resource equation (not using blocking) states:

**E = (total number of experimental units) – (number of treatment combinations)**

**E** was calculated for all experiments in the present study. Alcohol drinking, **E** = 28; Blood alcohol levels, **E** = 31; CPP, **E** = 31; LORR, **E** = 9; MD, **E** = 29; c-Fos, **E** = 18. According to Mead's rule, **E** should be between 10 and 20. If **E** is less than 10, increasing numbers would lead to good returns. If **E** is over 20, resources may be wasted.

There are some circumstances where it is justifiable for **E** to be greater than 20.

i. When experimental units are cheap and non-sentient.

ii. When the experiments are complex and involve several factors (such as treatments), both sexes, different time points, more than one genotype etc.

iii. When it is desirable that the numbers in each experimental sub group are balanced. This may not be possible if **E** is fixed. In such cases it may be acceptable for **E** to range up to 40 or more.

iv. When the aim of the experiment is the magnitude of a treatment effect, rather than the testing of a hypothesis i.e. that the treatment effect exists.

# 3. Results

## 3.1. Deficiency in αCaMKII autophosphorylation reduces alcohol drinking

In order to test a potential involvement of αCaMKII autophosphorylation in alcohol drinking behaviour a two-bottle free-choice paradigm was used. Mt and Ht animals drank significantly less alcohol than WT animals (Fig. 3.8A; ANOVA, genotype: $F_{2,124}$=11.17, p<0.0001; dose: $F_{3,124}$=55.10; p<0.0001; interaction: $F_{6,124}$=2.04, p=0.065). Planned pairwise comparisons showed that there were significant differences between Mt and WT animals at 8 vol.% (p=0.016) and 12 vol.% (p<0.0001). In addition, Ht alcohol consumption was reduced vs. WT at 12 vol.% (p=0.0002). Alcohol preference vs. water was significantly reduced in Mt compared to WT animals (Fig. 3.8B; genotype $F_{2,124}$=5.74, p=0.0041; dose: $F_{3,124}$=19.94, p<0.0001; interaction: $F_{6,124}$=1.50, p=0.18). Pairwise comparisons showed a significant difference between Mt and WT animals at 4 vol.% (p=0.022) and a tendency for 12 vol.% (p<0.058). These findings suggest that αCaMKII autophosphorylation may be required for the establishment of alcohol drinking and alcohol preference over water in a standard drinking test.

## 3.2. Repeated alcohol withdrawal eliminates αCaMKII autophosphorylation effects on drinking behaviour

Initial alcohol preference was reduced in Mt mice. This initial difference could be overcome by repeated withdrawal from alcohol. Voluntary alcohol consumption is known to escalate after repeated withdrawal periods, which is known as the alcohol deprivation effect (ADE; (Spanagel and Hölter, 2000). Mt as well as Ht animals consumed significantly less alcohol during baseline when offered a 16 vol.% alcohol solution (Fig. 3.8C; genotype $F_{2,124}$=11.67, p<0.0001; time: $F_{3,124}$=0.65; p=0.58; interaction: $F_{6,124}$=0.97, p=0.45). Planned pairwise comparisons vs. WT confirmed this (Mt: p<0.0001; Ht: p<0.0001). Alcohol preference vs. water was altered in Mt and Ht animals during baseline (Fig. 3.8D; genotype $F_{2,124}$=15.15, p<0.0001; time: $F_{3,124}$=1.93; p=0.13;

interaction: $F_{6,124}$=1.67, p=0.13). Planned pairwise comparisons vs. WT showed a reduction for Mt (p<0.0001) and Ht animals (p<0.0001).

After the first withdrawal period, alcohol consumption increased in the Mt and Ht animals across 4 days of reinstatement (genotype $F_{2,124}$=10.40, p<0.0001; time: $F_{3,124}$=2.59; p=0.056; interaction: $F_{6,124}$=2.20, p=0.048). However, Mt (p=0.0006) consumption was still below WT levels, the same was not true of Ht mice (p>0.05). Alcohol preference vs. water increased during this interval (genotype $F_{2,124}$=7.20, p<0.001; time: $F_{3,124}$=0.94; p=0.42; interaction: $F_{6,124}$=4.80, p=0.0002). Preference was still lower in Mt (p=0.007), but not in Ht mice (p>0.05) compared with WT animals.

After the second 3 week withdrawal period, consumption increased temporarily in all animals suggesting a clear ADE (genotype $F_{2,124}$=6.08, p=0.003; time: $F_{3,124}$=52.59; p<0.0001; interaction: $F_{6,124}$=1.76, p=0.11), interestingly Mt consumed alcohol at the same level as WT animals (p>0.05). While the ADE was comparable between all genotypes on day 1 of reinstatement of drinking behaviour, it decreased to a greater extent in the Ht animals from days 2-4 (p=0.0018). This effect was reflected when alcohol preference was measured (ANOVA; genotype $F_{2,124}$=7.67, p=0.0007; time: $F_{3,124}$=10.83; p<0.0001; interaction: $F_{6,124}$=0.41, p=0.87). While the second withdrawal increased absolute preference over water on day 1 of reinstatement, it eliminated the difference between Mt and WT animals (p>0.05). However, Ht animals still preferred alcohol less than WT animals (p=0.0012). Data suggest that the reduced alcohol preference in Mt mice can be overcome by repeated withdrawal. Upon reinstatement, alcohol consumption and preference increase to WT levels in the Mt mice. Findings might suggest that αCaMKII autophosphorylation may not control the capacity to consume alcohol, but controls the speed at which alcohol consumption is established.

### 3.3. Deficiency in αCaMKII autophosphorylation reduces sucrose preference

Alcohol consumption may be influenced in part by differences in taste perception. To control for altered taste sensitivity, preference for low and high concentrations of sucrose and avoidance of bitter tasting quinine were monitored. There was no genotype difference in the preference of 0.5% sucrose (Fig. 3.8E; $p>0.05$). However, a 5% sucrose solution was preferred less by the Mt, but not by Ht, mice (genotype: $F_{2,93}=6.20$, $p=0.003$; time: $F_{2,93}=0.76$; $p=0.47$; interaction: $F_{4,93}=0.31$, $p=0.87$). Pairwise comparisons between genotype groups showed a significant difference vs. WT ($p=0.002$). Quinine was less preferred than water by all mice. The 10 mg/dl concentration was avoided most by Ht animals (genotype: $F_{2,93}=10.38$, $p<0.0001$; time: $F_{2,93}=5.25$; $p=0.007$; interaction: $F_{4,93}=0.72$, $p=0.58$), which differed from WT ($p<0.014$). There was no genotype difference in the avoidance of a 20 mg/dl quinine solution ($p<0.05$). These data suggest that while taste sensitivity is not disturbed by a deficit in αCaMKII autophosphorylation, it may, nevertheless, reduce preference of sweet solutions at higher concentration. The degree of sucrose preference over water is an indicator of its hedonic value and rewarding properties (Papp et al., 1991). A reduced preference may therefore suggest that αCaMKII autophosphorylation is required for the establishment of the incentive properties of a consumed stimulus.

### 3.4. αCaMKII autophosphorylation has no effect on bioavailability of alcohol

In order to test whether αCaMKII autophosphorylation deficiency may change alcohol consumption by altering its bioavailability, blood alcohol levels were measured at three different time points after a 3.5 g/Kg alcohol i.p. injection. There was no effect of genotype ($F_{2,21}=0.38$, $p=0.69$) or time ($F_{2,21}=3.45$; $p=0.05$), nor interaction ($F_{4,21}=2.58$, $p=0.07$) on blood alcohol levels after a single alcohol injection (Fig. 3.8F). These findings suggest comparable bioavailability of alcohol in all genotypes.

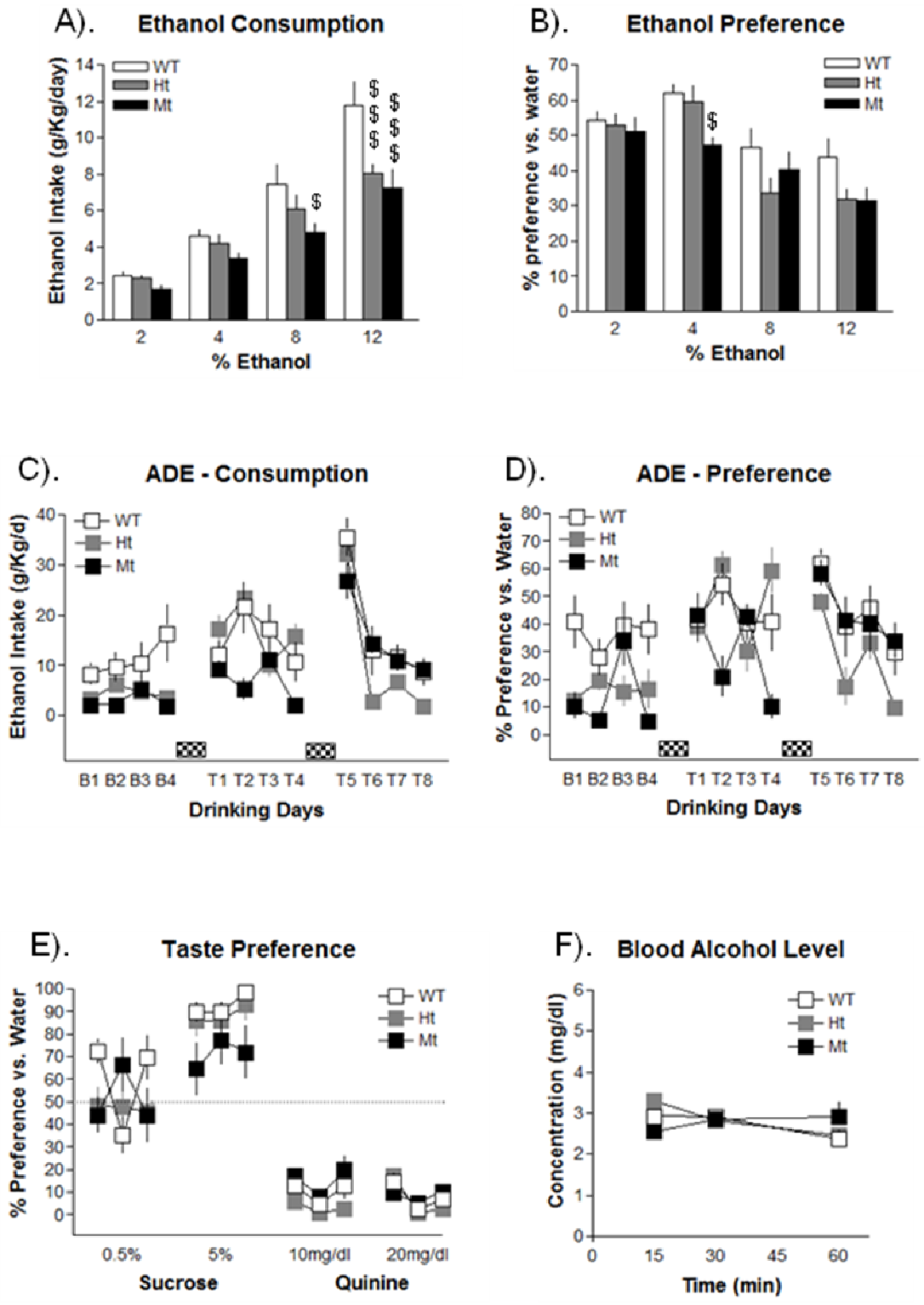



**Figure 3.8:** Alcohol drinking and blood alcohol levels. αCaMKII autophosphorylation deficient (Mt) mice initially drank less alcohol but eventually reach WT level after repeated withdrawal. Mice showed a largely preserved taste preference and avoidance and comparable alcohol bioavailability. (A) Mean alcohol consumption

measured over 4 days for each dose. (B) Mean alcohol preference vs. water measured over 4 days for each dose ($^{\$}$ $p<0.05$, $^{\$\$\$}$ $p<0.001$ vs. WT). (C) Mean consumption of a 16 vol. % alcohol solution per day. Alcohol deprivation effect on consumption after 2 three week withdrawal periods (hatched bars). (D) Mean preference vs. water of a 16 vol.% alcohol solution per day. Alcohol deprivation effect on preference after 2 three week withdrawal periods (hatched bars). (E) Mean preference of sucrose and quinine solution over water measured over three consecutive days, respectively. (F) Blood alcohol levels (mean $\pm$SEM) after a 3.5 g/Kg (i.p.) injection of alcohol. For more statistical details: see main text.

### 3.5. αCaMKII autophosphorylation controls speed of alcohol-induced CPP establishment and extinction

αCaMKII deficiency affects learning processes by delaying the speed, but not the capacity, at which new behaviors are acquired (Giese *et al.*, 1998; Irvine *et al.*, 2005). The motivational properties of alcohol in a CPP paradigm and the speed of CPP establishment were assessed. Alcohol-induced CPP is established faster in Mt mice than in WT mice (Fig. 3.9A). Two-way ANOVA revealed a significant test effect ($F_{4,136}$= 8.19, p=0.001) and significant test x genotype effect ($F_{8,136}$=2.82, p=0.006). Planned pairwise comparisons show that WT mice establish alcohol-induced CPP at preference test 3 (p<0.001), i.e. after seven conditioning trials. This preference is maintained after an incubation period of 7 days (p=0.005). In contrast, CPP is established in Mt mice after only a single conditioning trial, at preference test 1 (p=0.002). Mt mice maintain this high preference (test 2, p<0.001; test 3, p=0.002; incubation test, p=0.001) through-out the experiment. Interestingly, Ht mice do not develop a significant CPP (p>0.05).

Two-way ANOVA for the extinction (Fig. 3.9B) of a preference for the alcohol-paired compartment revealed a significant test effect ($F_{5,170}$=6.38, p<0.001) and test x genotype interaction ($F_{10,170}$=2.66, p=0.004). Pairwise comparisons suggest that WT CPP does not extinguish much compared to baseline (test 1, p=0.01; test 2, p=0.008; test 3, p=0.005; test 4, p=0.03). There was an enhancement of CPP observed in WT mice (p<0.001) after reinstatement. Mt

mice maintain CPP after 3 extinction trials (test 1, $p<0.001$; test 2, $p<0.001$), an effect which disappears after preference test 3 ($p>0.05$) when Mt mice appear to have no preference for the alcohol-paired compartment over the saline-paired compartment. A CPP was observed in preference test 4 ($p=0.02$), although less pronounced than before. The CPP was enhanced after a further reinstatement conditioning trial ($p=0.03$). Ht mice did not develop CPP and therefore could not extinguish the behaviour. However, after a single reinstatement conditioning trial, Het showed a significant CPP ($p=0.02$).

### 3.6. Alcohol administration produces an acute sedating effect in αCaMKII autophosphorylation-deficient mice, but not in wild-types

Activity in the alcohol-paired compartment during all conditioning trials (Fig. 3.9C) showed a significant test effect ($F_{7,238}=26.25$, $p=0.001$), and a significant test x genotype interaction effect ($F_{14,238}=7.11$, $p<0.001$). WT locomotor response to alcohol does not diminish compared to baseline after alcohol injection. However, a slight decline in locomotion was observed after the 3rd ($p=0.004$), 5th ($p=0.05$), 6th ($p=0.04$) and 7th ($p=0.001$) alcohol injection. Ht mice showed no reduction in activity levels after the first injection, but did show diminished locomotor activity after 2nd ($p=0.03$), 3rd ($p<0.001$), 5th ($p<0.001$), 6th ($p=0.008$), and 7th ($p<0.001$) alcohol injection. At baseline, Mt mice responded in a hyperactive manner to the test arena when compared to WT animals. However, after a single alcohol injection, Mt mice show a significant sedating effect on locomotor response ($p<0.001$), an effect which was maintained through-out all remaining conditioning trials (all: $p<0.001$). These results suggest that alcohol reduces the hyperactivity that may be induced by novel threatening situations (Easton *et al.*, 2011; Chapter 2) in the Mt mice. As such, the fast CPP establishment in the Mt mice may be due to fast acting, highly persistent, anxiolytic and negative reinforcing effect of the alcohol.

### 3.7. Conditioned sedation effects in αCaMKII autophosphorylation-deficient mice

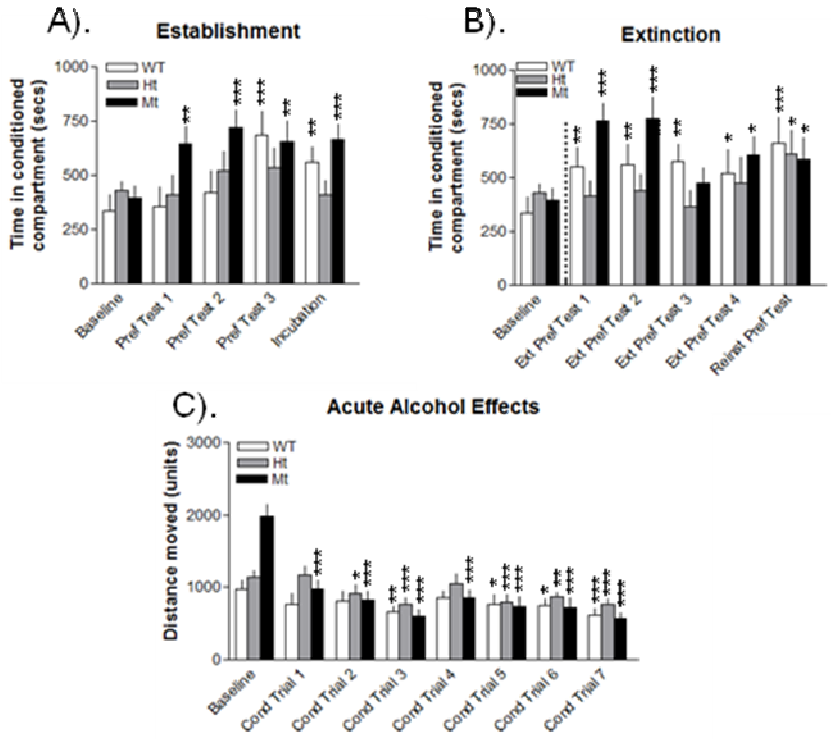
In Mt mice, there was a conditioned sedation effect observed during repeated testing. Two-way ANOVA showed a significant test effect ($F_{4,136}$=9.47, p<0.001) and test x genotype interaction ($F_{8,136}$=7.86, p<0.001) for conditioned locomotor effects in the alcohol compartment for CPP establishment (Fig. 3.9D). Similar effects are seen during extinction (Fig. 3.9E; test: $F_{5,170}$=8.77, p<0.001; test x genotype interaction: $F_{10,170}$=4.91, p<0.001). There are no conditioned locomotor effects seen in the Ht group. WT mice did not establish a conditioned sedation effect until preference test 3 (Fig. 3.9D; p=0.03). This effect was lost after the incubation period (p>0.05). Activity levels in WT mice remain at baseline through-out the extinction procedure (Fig. 3.9E), with a slight decrease in activity after extinction preference test 4 (p=0.008). Mt mice, however, show a robust decline in locomotor activity in the alcohol-paired compartment (Fig. 3.9D), becoming more in line with the response of the WT mice. This effect is stable across all trials (all: p<0.001). The sedation effect does not extinguish after repeated extinction trials and the reinstatement preference test (Fig. 3.9E; all: p<0.001).

### 3.8. Habituation effects cannot account for the decrease in activity seen in response to alcohol in αCaMKII autophosphorylation-deficient mice

Activity levels in the saline compartment during preference tests in CPP establishment (Fig. 3.9F) showed a significant test effect ($F_{4,136}$=3.61, p=0.007). Activity during CPP extinction reveals significant genotype ($F_{2,34}$=6.81, p=0.003) and test effects ($F_{5,170}$=2.74, p=0.02). WT mice showed an initial decrease in activity in response to saline treatment (Fig. 3.9F; test 1: p=0.02; test 2: p=0.03). However, levels return to baseline after preference test 3 (p>0.05). Activity levels are not significantly different from baseline for the majority of the extinction procedure (Fig. 3.9G), with a slight decrease in response after preference test 4 (p=0.009) and the reinstatement test (p=0.01). Mt activity levels slowly declined in the saline compartment during CPP establishment (Fig. 3.9F; test 3: p<0.001). This decrease is not as great

as the effect seen after alcohol administration. It is therefore unlikely that basic habituation effects account for the reduction in locomotor activity in response to alcohol. This basal activity response is maintained through-out the extinction procedure (Fig. 3.9G).

## Conditioned Place Preference




## Conditioned Locomotor Effects

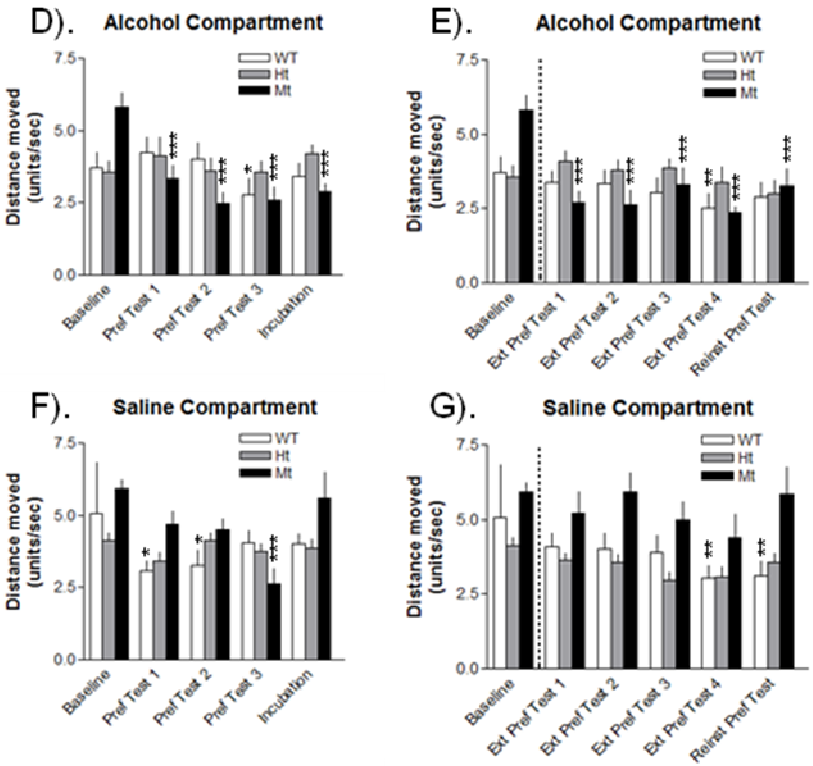


**Figure 3.9:** Conditioned Place Preference. The anxiolytic/negative reinforcing properties of alcohol may account for the accelerated establishment of alcohol-induced conditioned place preference (CPP) in Mt mice compared to WT mice. Time spent in the conditioning (alcohol paired) compartment during the establishment of CPP (A) and extinction of place preference (B). Distance travelled during the first 5 minutes of baseline pre-test, and in alcohol-paired 5 minute conditioning trials (C) compared to baseline. Conditioned locomotor effects of alcohol during CPP testing for the alcohol-paired compartments (D,E) and saline-paired compartments (F,G). Planned pairwise comparisons to baseline, *p<0.05, **p<0.01, ***p<0.001.

### 3.9. No role of αCaMKII autophosphorylation in the hypnotic effects of alcohol

There was no difference in the hypnotic effects of alcohol between groups of alcohol naïve mice following an acute alcohol injection ($p>0.05$; Fig. 3.10A). After subchronic alcohol treatment (7 daily alcohol injections) Ht mice were less sensitive to the hypnotic properties of alcohol (time: $F_{2,12}=12.19$, $p=0.004$; Fig. 3.10A) and were significantly more tolerant to the hypnotic effects of alcohol than both Mt ($p=0.04$) and WT mice ($p=0.02$; Fig. 3.10A). Figure 3.10B illustrates the amount of time mice spent in an ataxic state as a result of a high alcohol dose. This was not different between groups of alcohol naïve mice following an acute alcohol injection ($p>0.05$). After alcohol treatment increased, Mt ($p=0.04$) and Ht ($p=0.04$) alcohol-experienced mice were significantly more tolerant to the ataxic effects induced by a high alcohol dose, thus spending less time in an ataxic state (time: $F_{1,12}=9.97$, $p=0.008$; Fig. 3.10B). This effect was not seen in WT animals ($p>0.05$; Fig. 3.10B).

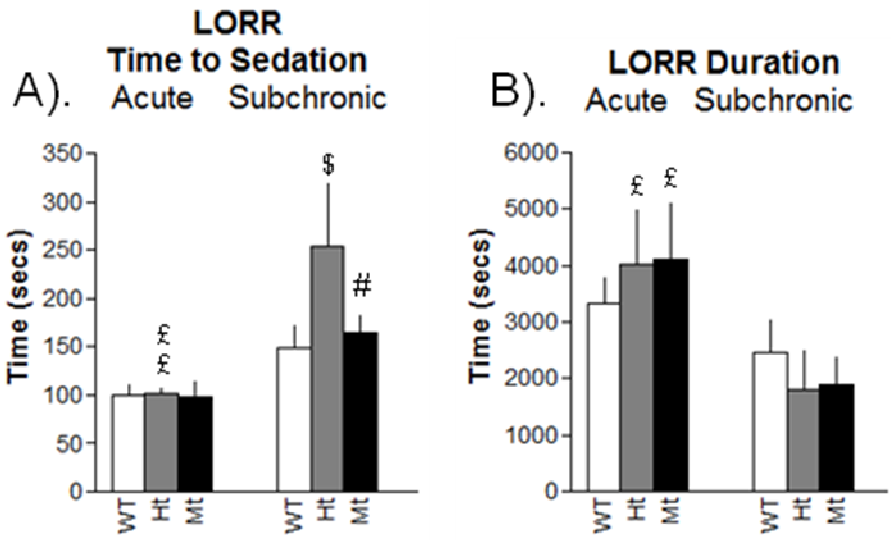



Figure 3.10: Loss of Righting Reflex. The hypnotic effects of alcohol were experienced similarly by αCaMKII autophosphorylation deficient, Ht and WT alcohol naïve mice. Loss of righting reflex (LORR) to sedation (A) and LORR duration (B) after acute and subchronic (after seven prior alcohol treatments) alcohol (3.5g/Kg, i.p.) treatment. Planned pairwise comparisons: $^{\$}p<0.05$ vs. WT; $^{\#}p<0.05$ vs. Ht; $^{£}p<0.05$ $^{££}p<0.01$, acute genotype group v subchronic genotype group.

### 3.10. Acute locomotor activating effects of alcohol are absent in αCaMKII autophosphorylation deficient mice

In contrast to the relatively new environment of a CPP box, alcohol naïve mice showed no differences between genotypes in locomotor activity in a well habituated environment of the in-vivo microdialysis test ($p>0.05$; Fig. 3.11A). An acute alcohol injection increased locomotor activity in WT, but not in Mt or Ht mice. Although two-way ANOVA failed to show significant genotype effects or interactions ($p>0.05$), pairwise comparisons revealed a significant difference between Mt and WT mice 60 minutes after acute alcohol injection ($p=0.03$; Fig. 3.11B).

Basal (no alcohol on board) behavioural activation levels of Mt mice changed after alcohol pre-treatment (subchronic exposure), and showed a significant increase in basal activation ($p<0.05$; Fig. 3.11A). Subchronic Mt activation levels were also elevated compared to alcohol experienced WT (<0.0001) and

Ht (<0.001) mice. Following seven prior daily alcohol treatments, two-way ANOVA revealed a further alcohol injection produced a significant increase in activity in WT and Ht but not Mt mice (time: $F_{11,231}$=2.97, p=0.001; interaction: $F_{22,231}$=1.83, p=0.02; Fig. 3.11C). Pairwise comparisons showed a significant difference between groups at 20 minutes (Mt vs. WT p=0.05) and 160 minutes (WT vs. Ht p=0.04) post injection. Data suggest that the acute locomotor activating effects of alcohol are absent in Mt mice following both acute and subchronic exposure.

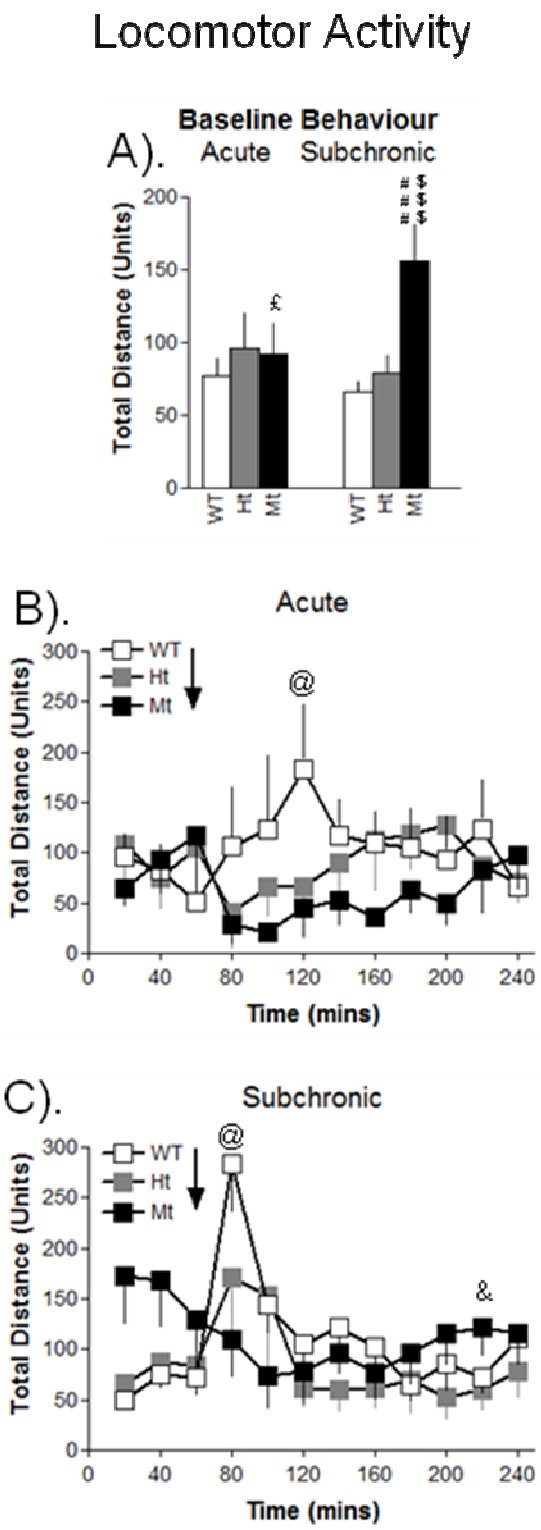



**Figure 3.11: Locomotor Activity.** The effects of an alcohol (2 g/Kg, i.p.) injection on αCaMKII autophosphorylation deficient and wild-type mice in a well habituated (>2h before baseline) open field. Baseline behavioural activity was not different between genotypes; Planned pairwise comparisons: $^{\$\$\$}$p<0.001 vs. WT; $^{\#\#\#}$p<0.001 vs. Ht;

[£]p<0.05 acute genotype group v subchronic genotype group **(A)**. Locomotor activity recorded after acute **(B)** and subchronic (seven) alcohol treatments **(C)**. Arrow indicates time of alcohol injection. Planned pairwise comparisons using Fisher's LSD tests: [@]p<0.05 Mt vs. WT; [&]p<0.05 WT vs. Ht.

### 3.11. There is no difference in basal DA levels in the NAcc and the PFC in alcohol naïve αCaMKII autophosphorylation deficient and wild type mice.

Neurotransmitter levels were measured in the NAcc and PFC in alcohol naïve αCaMKII autophosphorylation deficient (Mt), Ht and WT mice, prior to an acute alcohol challenge. There were no differences in DA levels in the NAcc (Fig. 3.12A) or PFC (Fig. 3.12B) between genotype groups prior to alcohol administration in alcohol naïve animals. Alcohol naïve Het mice were shown to have elevated 5-HT levels in both the NAcc and PFC. Ht NAcc levels ($F_{2, 88}$=11.67, p<0.001; Fig. 3.12C) were significantly different compared to WT (p<0.001) and Mt (p<0.001) mice. In the PFC ($F_{2, 89}$=17.56, p<0.001; Fig. 3.12D) Ht 5-HT levels were also significantly different compared to both WT (p<0.001) and Mt (p<0.001) mice. NA levels in the NAcc were not significantly different between genotype groups (Fig. 3.12E). However, In the PFC ($F_{2, 90}$=4.93, p=0.009; Fig. 3.12F) Mt were found to have significantly higher NA levels than both WT (p=0.02) and Ht (p=0.004) mice.

### 3.12. NAcc DA levels are elevated after subchronic alcohol treatment in αCaMKII autophosphorylation deficient mice.

Neurotransmitter levels were measured in the NAcc and PFC in alcohol experienced αCaMKII autophosphorylation deficient (Mt), Ht and WT mice, prior to an alcohol challenge. After 7 previous alcohol treatments, there were no differences in basal DA levels between genotype groups in the NAcc. However, planned pairwise comparisons revealed a significant increase overtime in Mt (p=0.002; Fig. 3.12A), but not in Ht or WT mice. In the PFC there was a difference in basal DA levels seen between alcohol experienced

groups ($F_{2, 68}$=4.08, p= 0.02; Fig. 3.12B), where Ht were significantly different from WT (p=0.02) and Mt (p=0.01) mice, but Mt and WT mice were not significantly different from one another. Planned pairwise comparisons revealed a significant increase overtime in Ht (p=0.02; Fig. 3.12B), but not in Mt or WT mice. The degree of alcohol exposure did not affect resting 5-HT levels in the NAcc and PFC. Alcohol experienced Ht mice were shown to have elevated basal 5-HT levels in both the NAcc and PFC. NAcc levels ($F_{2, 79}$=11.51, p<0.001; Fig. 3.12C) were significantly different from WT (p<0.001) and Mt (p<0.001) mice. In the PFC ($F_{2, 75}$=11.40, p<0.001; Fig. 3.12D) 5-HT levels were also significantly different compared to both WT (p<0.001) and Mt (p<0.001) mice. These effects of genotype were not influenced by the degree of alcohol exposure. Subchronic alcohol treatment was seen to affect basal NA levels in the NAcc ($F_{2, 80}$=3.75, p=0.03; p<0.001 WT vs, Ht; Fig. 3.12E), with an observed increase in NAcc resting NA levels after subchronic alcohol administration in WT mice (p<0.001; Fig. 3.12E) only. In the PFC, Ht mice had decreased resting NA levels after subchronic alcohol treatment ($F_{2, 72}$=5.07, p=0.008; Fig. 3.12F) compared to WT (p=0.002) and Mt (p=0.04) mice. The increase in WT NA levels in response to alcohol administration between naïve and alcohol experienced mice was significantly different after several alcohol treatments (p<0.001).

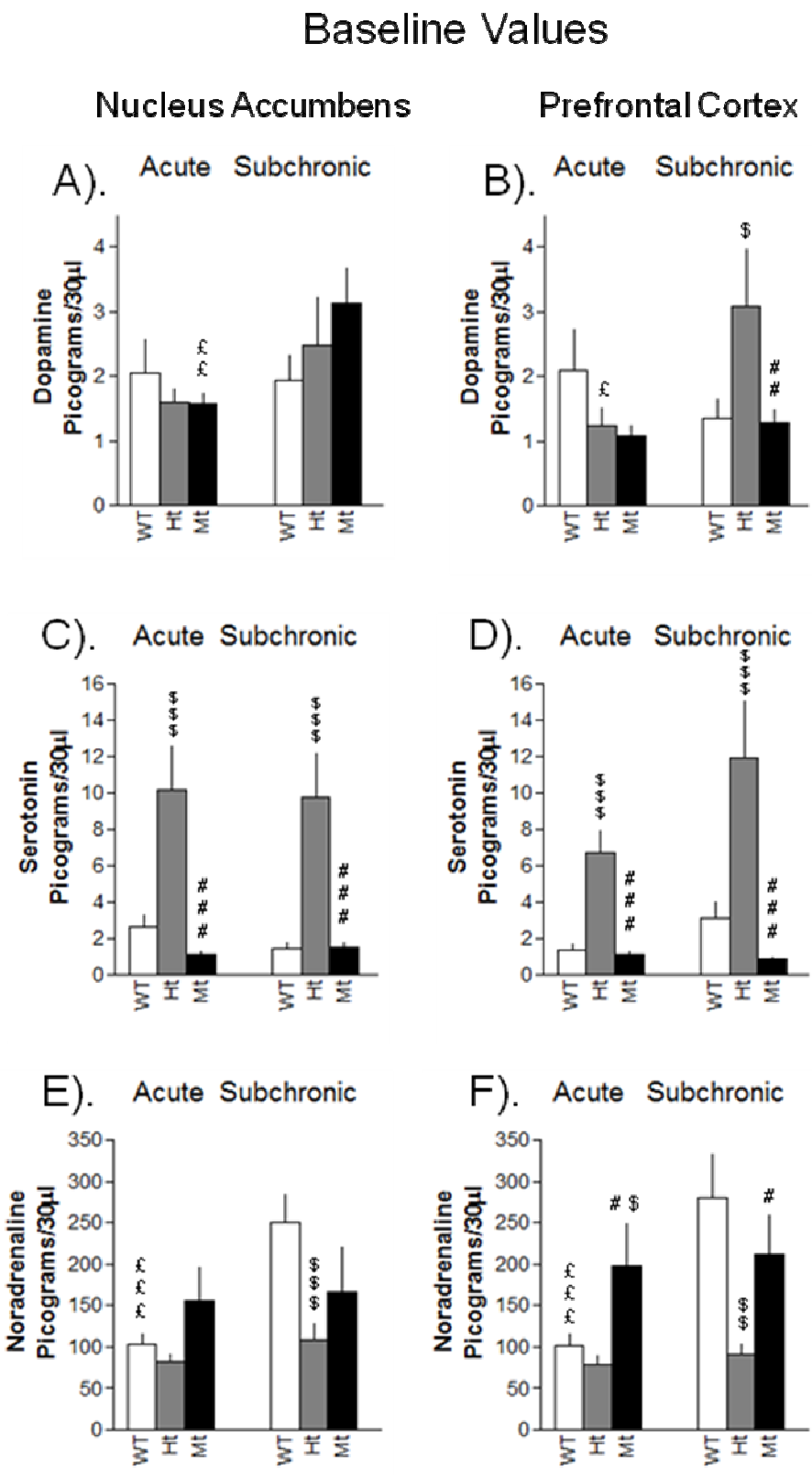



**Figure 3.12:** Baseline monoamine levels adapted after subchronic (seven prior alcohol treatments) alcohol exposure. Bars represent neurotransmitter levels in the

NAcc prior to and after alcohol treatment for DA (A); 5-HT (C); and NA (E). Baseline neurotransmitter levels in the PFC prior to and after alcohol treatment for DA (B); 5-HT (D); and NA (F). Planned pairwise comparisons: $^{\$}p<0.05$ ,$^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs. WT; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. Ht; $^{£}p<0.05$, $^{££}p<0.01$, $^{£££}p<0.001$, acute genotype group v subchronic genotype group.

### 3.13. The acute dopaminergic effects of alcohol in the NAcc are absent in αCaMKII autophosphorylation deficient mice

αCaMKII autophosphorylation may control the incentive properties of alcohol. Results from alcohol drinking experiments suggest that neurochemical markers for the incentive and behaviourally activating properties of alcohol would be reduced in Mt mice after initial exposure. Given that alcohol drinking reached WT level after subchronic exposure, neurochemical responses to alcohol might be expected to normalize to WT levels with repeated exposure. Acute alcohol administration increased extracellular DA levels in the NAcc of WT mice. This alcohol-induced DA increase was absent in Mt and Ht mice. Two-way ANOVA showed a significant genotype ($F_{2,26}=4.20$, $p=0.03$) and time effects ($F_{11,286}=2.21$, $p=0.01$), but no significant genotype x time interaction ($p>0.05$; Fig. 3.13A). Planned pairwise comparisons revealed a significant alcohol-induced increase in DA levels in the NAcc in WT mice only (40 mins: WT vs. Ht, $p=0.02$; 80 mins: WT vs. Mt, $p=0.01$; 100 mins: WT vs. Mt, $p=0.01$ and WT vs. Ht, $p=0.01$; 120 mins: WT vs. Mt, $p=0.04$ and WT vs. Ht, $p=0.02$; Fig. 3.13A). DA levels in the PFC of all mice did not change in response to an acute alcohol challenge (Fig. 3.13C) despite a significant genotype x time interaction, as revealed by two-way ANOVA ($F_{22,286}=1.70$, $p=0.03$).

### 3.14. Subchronic alcohol treatment reduces DA response differences between genotype groups in the NAcc

After subchronic alcohol exposure for 7 days, an acute alcohol injection led to a slight increase in NAcc DA levels in WT mice, which was far less pronounced than in naïve animals. There was no such effect in Mt or Ht mice

(Fig. 3.13B). Two-way ANOVA did not show significant genotype effects or an interaction ($p<0.05$). However, planned pairwise comparisons revealed significant group differences at 100 minutes (Mt vs. WT $p=0.02$) and 140 minutes (Mt vs. Ht $p=0.02$) post alcohol administration. Data suggest that after several alcohol pre-treatments, the NAcc DA response is greatly diminished in WT mice, remaining unchanged in Mt and Ht groups, after a further alcohol challenge, thus reducing response differences between genotype groups. Subchronic DA levels in the PFC were not significantly different between groups ($p>0.05$; Fig. 3.13D).

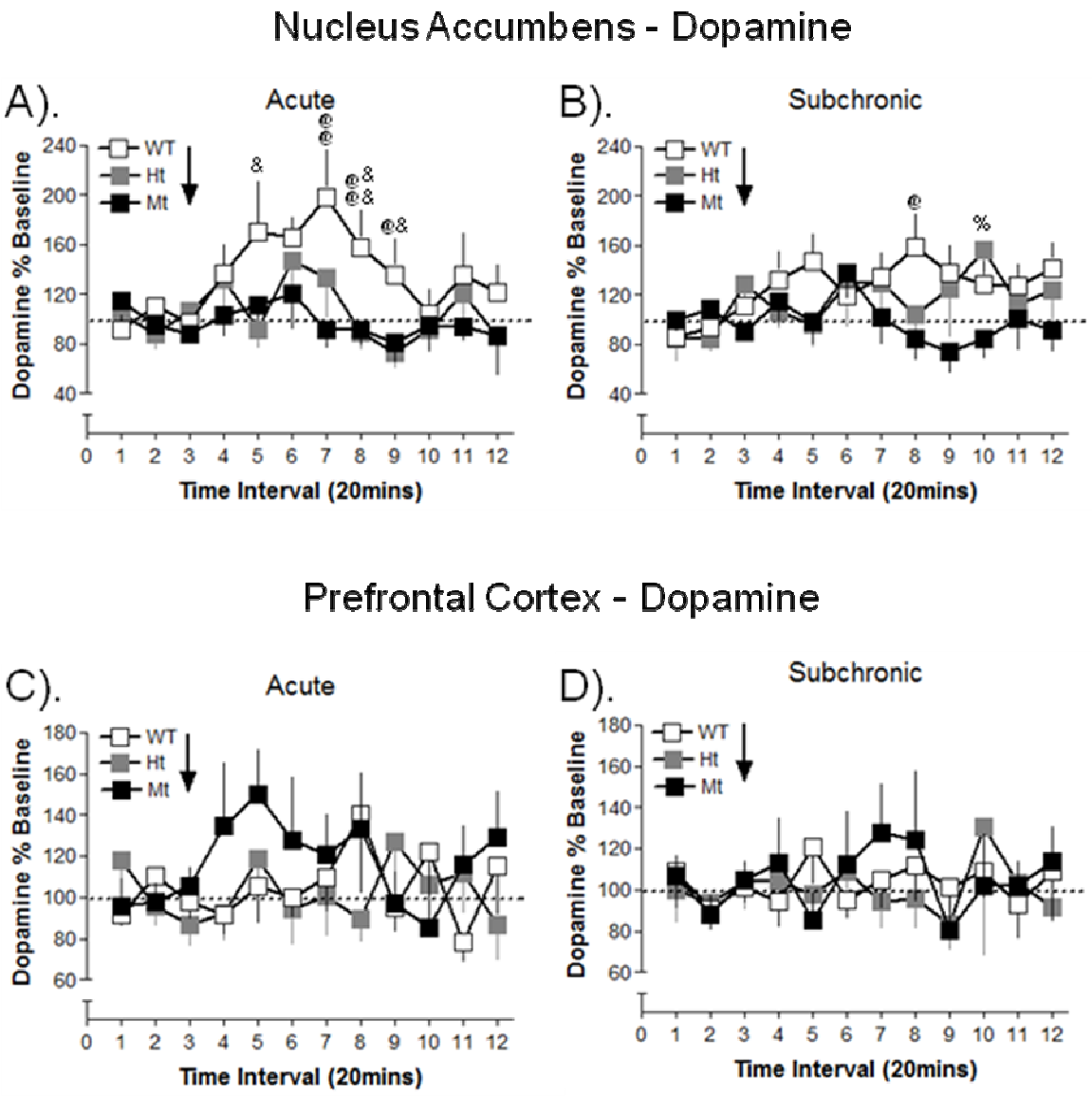



**Figure 3.13: In vivo microdialysis dopamine levels.** The acute dopaminergic effects of alcohol in the nucleus accumbens (NAcc) were absent in αCaMKII autophosphorylation deficient mice. Differences between genotype groups diminish over time and after increased alcohol exposure. Extracellular dopamine (DA) levels in the NAcc after acute **(A)** and subchronic (following seven prior alcohol treatments) **(B)** alcohol treatments as percent of baseline. Extracellular DA levels in the prefrontal cortex (PFC) after acute **(C)** and subchronic **(D)** alcohol treatments. Arrow indicates time of alcohol injection. Planned pairwise comparisons using Fisher's LSD tests between groups at each time point: $^{@}p<0.05$, $^{@@}p<0.01$ MT vs. WT; $^{\&}p<0.05$, $^{\&\&}p<0.01$ Ht vs. WT; $^{\%}p<0.05$ MT vs. Ht.

### 3.15. Deficiency in αCaMKII autophosphorylation leads to an increased 5-HT response in the PFC after acute alcohol treatment

An initial difference in alcohol consumption/preference, which normalizes with repeated exposure, may be due to a deregulation in the serotonergic system in Mt mice with potential neurochemical differences normalizing to WT levels after subchronic exposure. To test this hypothesis extracellular 5-HT levels were monitored after acute alcohol exposure and after repeated exposure. After the first alcohol treatment, 5-HT in the NAcc was not significantly different between groups ($p>0.05$). Two-way ANOVA revealed a significant time effect ($F_{11,308}=2.02$, $p=0.03$; Fig. 3.14A), as a gradual reduction in 5-HT was observed over time in all genotype groups. In WT and Ht animals, there was no effect of acute alcohol administration on 5-HT levels in the PFC. However, in Mt mice, alcohol led to an increase in 5-HT. A two-way ANOVA showed a significant genotype x time interaction ($F_{22,319}=2.22$, $p=0.001$; Fig. 3.14C). Planned pairwise comparisons indicate significant differences between Mt and the other genotype groups at 20 minutes (Mt vs. WT $p=0.008$; Mt vs. Ht $p=0.02$) and 40 minutes (Mt vs. WT $p<0.001$; Mt vs. Ht $p=0.002$) post alcohol administration.

### 3.16. Subchronic alcohol treatment does not alter the serotonergic response to alcohol in the PFC

After repeated alcohol treatments, a further acute alcohol challenge does not alter serotonergic response in the NAcc ($p>0.05$; Fig. 3.14B). In the PFC, there was no effect of alcohol on 5-HT levels in WT or Ht animals, but there was a small, non-significant increase in Mt mice (Fig. 3.14D). Although two-way ANOVA did not find significant genotype or interaction effects ($p>0.05$), pairwise comparisons between groups revealed significant differences at 40 minutes (Mt vs. Ht $p=0.04$), 120 minutes (WT vs. Mt $p=0.05$) and 160 minutes (WT vs. Mt $p=0.02$; Mt vs. Ht $p=0.03$) post alcohol injection.

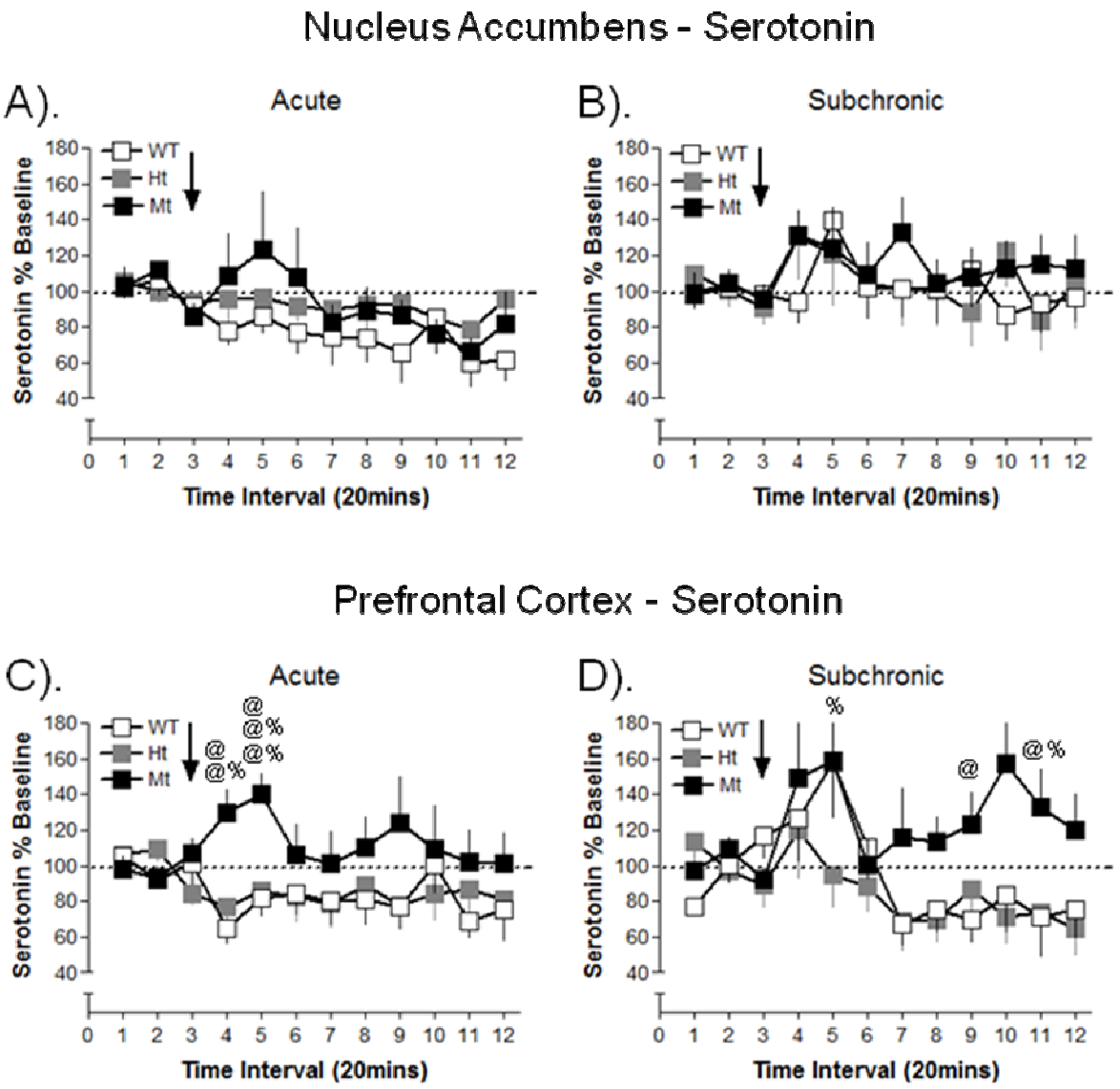



**Figure 3.14:** In vivo microdialysis serotonin levels. Serotonergic responses to alcohol in the nucleus accumbens (NAcc) and prefrontal cortex (PFC) revealed a deregulation of the reward system. The response remains unchanged after several alcohol pre-treatments. Extracellular serotonin (5-HT) levels in the NAcc after acute (A) and subchronic (following seven prior alcohol treatments) (B) alcohol treatments as percent of baseline. Extracellular 5-HT levels in the PFC after acute (C) and subchronic (D) alcohol treatment. Arrow indicates time of alcohol injection. Planned pairwise comparisons using Fisher's LSD tests between groups at each time point, $^{@}p<0.05$, $^{@@}p<0.01$, $^{@@@}p<0.001$ MT vs. WT; $^{\%}p<0.05$, $^{\%\%}p<0.01$ = MT vs. Ht.

### 3.17. The acute noradrenergic effects of alcohol in the NAcc are absent in αCaMKII autophosphorylation deficient mice

Deficiency in αCaMKII autophosphorylation led to a deregulation of noradrenergic responses to alcohol in the NAcc and the PFC. In the NAcc, alcohol induced a gradual increase in extracellular NA levels. This effect was attenuated in Mt mice and absent in the Ht mice. Two-way ANOVA showed a significant time effect ($F_{11,341}$=2.27, p=0.01) and genotype x time interaction ($F_{22,341}$=1.69, p=0.03; Fig. 3.15A). Pairwise comparisons revealed a significant difference between genotype groups 40 minutes (WT vs. Ht p=0.04), 60 minutes (WT vs. Ht p=0.05), 140 minutes (WT vs. Mt p=0.05; WT vs. Ht p=0.02) and 160 minutes (WT vs. Mt p=0.02; WT vs. Ht p=0.008) post alcohol administration. In the PFC, acute alcohol had no effect on NA levels (p>0.05; Fig. 3.15C).

### 3.18. Subchronic alcohol treatment alters acute NA responses

After subchronic alcohol treatment, an acute alcohol injection no longer had an effect on extracellular NA levels in the NAcc (time: $F_{11,286}$=2.67, p=0.002; pre-planned: p>0.05; Fig. 3.15B) and there were no longer any significant differences between groups (p>0.05). After subchronic alcohol treatment, an acute alcohol injection caused an increase of PFC NA levels in WT, but not in Mt or Ht mice (Fig. 3.15D). While an ANOVA failed to yield a significant genotype or interaction effect (p>0.05), planned pairwise comparisons revealed WT NA levels to be significantly different from Ht and Mt groups 20 minutes (WT vs. Mt p=0.05), 40 minutes (WT vs. Mt p=0.04), 80 minutes (WT vs. Mt p=0.02; WT vs. Ht p=0.04), 100 minutes (WT vs. Ht p=0.03) and 140 minutes (WT vs. Mt p=0.009; WT vs. Ht p=0.03) after alcohol administration.

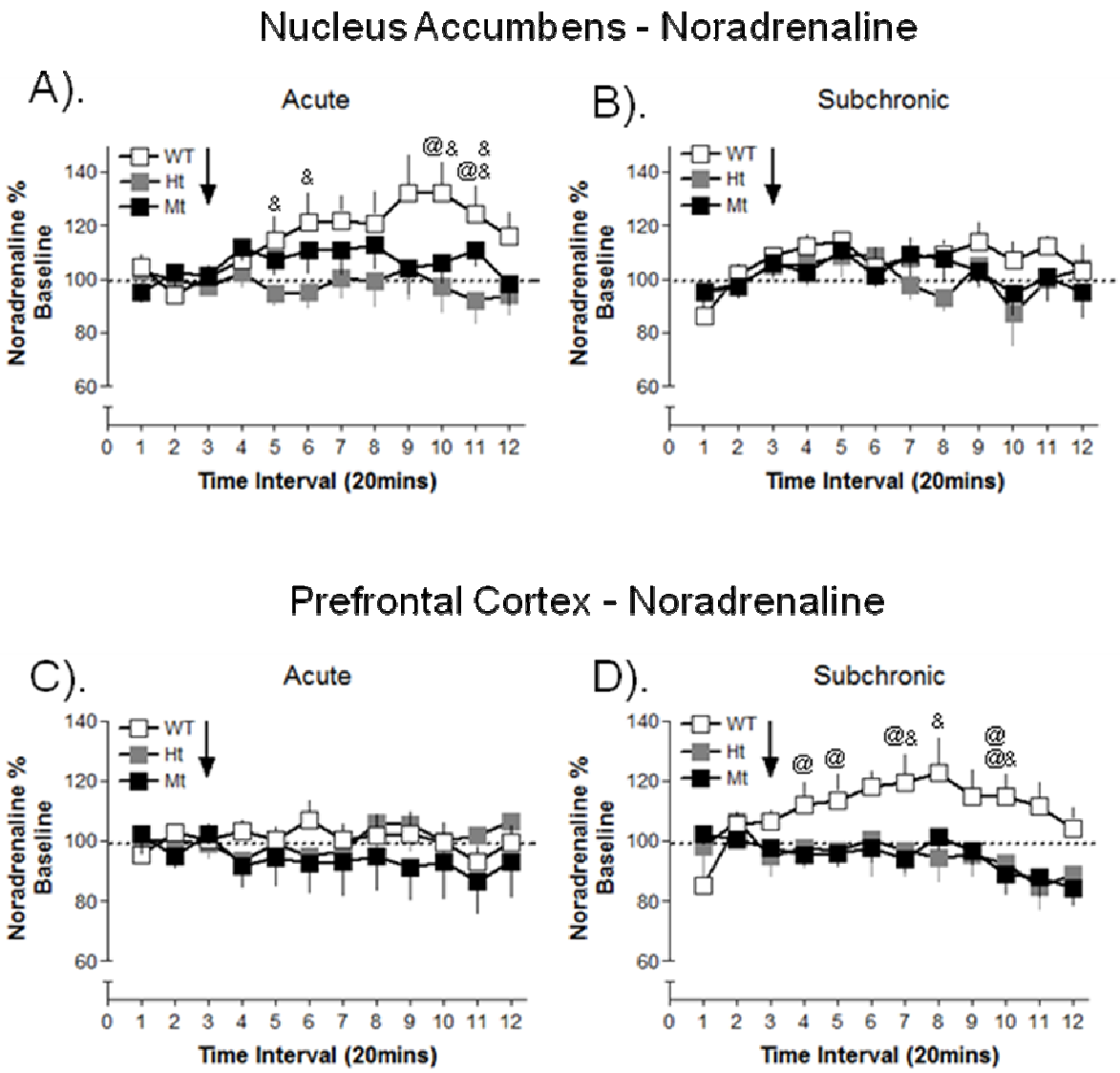



**Figure 3.15:** In vivo microdialysis noradrenaline levels. Alcohol increased noradrenaline (NA) levels in the nucleus accumbens (NAcc) of wild-type (WT), but not αCaMKII autophosphorylation deficient (Mt) or heterozygous (Ht) mice. Extracellular NA levels in the NAcc after acute (A) and subchronic (following seven prior alcohol treatments) (B) alcohol treatments as percent of baseline. Extracellular NA levels in the prefrontal cortex (PFC) after acute (C) and subchronic (D) alcohol treatments. Arrow indicates time of alcohol injection. Planned pairwise comparisons using Fisher's LSD tests between groups at each time point, $^{@}p<0.05$, $^{@@}p<0.01$ MT vs. WT; $^{\&}p<0.05$, $^{\&\&}p<0.01$ WT vs. Ht.

### 3.19. αCaMKII autophosphorylation-deficient mice show enhanced c-Fos activation in the rostral but not caudal VTA following alcohol exposure

The effects of acute and subchronic alcohol administration on c-Fos activation in the rostral and caudal VTA of Mt and WT mice (Fig. 3.16) were measured in order to determine the origin of altered DA responses to alcohol. Mt mice showed an increased c-Fos expression in the rostral part of the VTA after both acute ($F_{1,10}$=6.32, $p<0.05$) and subchronic ($F_{1,9}$=20.7, $p<0.01$) alcohol treatment. There was no significant difference between Mt and WT animals in c-Fos expression in the caudal part of the VTA under both treatment conditions ($p>0.05$).

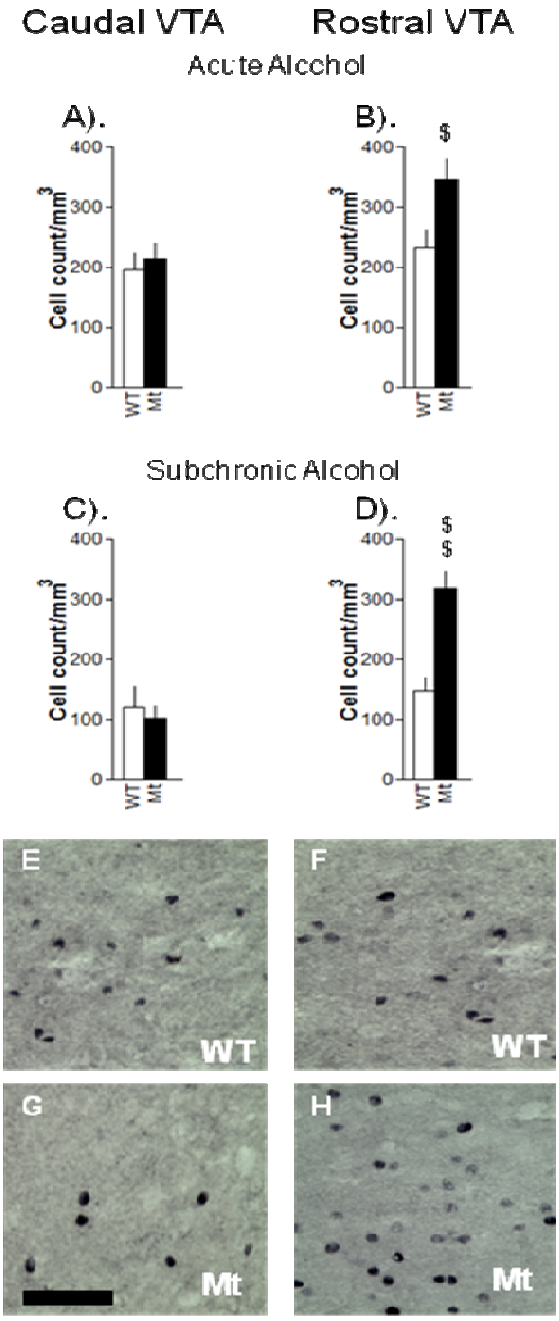



**Figure 3.16:** c-Fos activation. αCaMKII autophosphorylation deficient mice showed increased c-Fos expression in the rostral, but not caudal, ventral tegmental area (VTA) after both acute and subchronic alcohol treatment. c-Fos labelling of the caudal (A, C, E, G) and rostral (B, D, F, H) VTA after acute or 7 days subchronic alcohol (2 g/Kg, i.p.) treatment. C-Fos was determined 70 min after last alcohol injection (t-tests, $^{\$}p<0.05$ vs. WT, $^{\$\$}p<0.01$ vs. WT). Panels E-H show c-Fos labelled cells of the VTA after single acute alcohol treatment (bar=100 µm).

## 4. Discussion

αCaMKII is implicated in addictive pathways and alcohol, in particular, can alter αCaMKII expression levels in the brain after chronic alcohol exposure (Mahadev *et al.*, 2001). However, the specific role of αCaMKII and related molecular mechanisms are unclear. The present study reported that αCaMKII$^{T286A}$ Mt mice self-administer alcohol significantly less, and prefer alcohol less, than WT animals especially at higher percent alcohol solutions. This difference was no longer observed when mice were taken through repeated periods of withdrawal and reinstatement. Taste sensitivity remained largely unaffected, though a reduction in the incentive properties of a sucrose solution was observed in Mt mice. Blood alcohol levels were found to be comparable across genotype groups, thereby ruling out a difference in the bioavailability of alcohol as the cause of the altered alcohol consumption rates observed in Mt mice. These data suggest that αCaMKII autophosphorylation may control the speed at which a preference for alcohol is established, but not the capacity to consume alcohol.

The CPP test is designed to assess the motivationally rewarding properties of an alcohol associated cue (Cunningham *et al.*, 2006). Present data showed that αCaMKII autophosphorylation may control the speed at which alcohol-induced CPP was established and extinguished. Preference for the alcohol-paired compartment was established after seven conditioning trials in WT mice, which is in line with classical alcohol CPP establishment (Risinger and Oakes, 1996; Liu *et al.*, 2008). Rather unexpectedly, Mt mice established a profound alcohol CPP after a single alcohol conditioning trial, despite the fact that alcohol drinking behaviour was seen to develop at a slower rate in Mt compared to WT mice. The disparity between the establishment of alcohol drinking and CPP may be explained by considering the acute effects of alcohol in the CPP paradigm. Previous work (Easton *et al.,* 2011; Chapter 2) shows that αCaMKII$^{T286A}$ Mt mice have heightened activity in response to potentially threatening situations and environments while showing no altered behaviour in well-habituated environments (Easton *et al.*, 2011). Arguably one key component to compulsive drug seeking is the alleviation of a negative affective state by a negative reinforcement

mechanism (Koob, 2011). Alcohol drinking behaviour was measured in a familiar homecage environment. In contrast, CPP rewarding effects were measured in a less familiar environment, which might together with the injection process represent an aversive or threatening procedure, although this was not empirically tested. This view was supported by the observed hyperactivity in Mt mice showed in both the CPP baseline trial in this study and in a novel open field, but not in familiar home cages (Easton *et al.*, 2011). In contrast to WT mice, 2g/kg alcohol had an acute and persistent sedating effect in the αCaMKII autophosphorylation-deficient mice. One possible explanation of this effect is that the alcohol alleviated the threat-induced behavioural responses in Mt mice in a potentially aversive novel test situation. Furthermore, a single alcohol pairing was sufficient to induce a significant conditioned sedation effect in the Mt, but not in WT or Ht mice. Therefore, it is possible that the reduction of an aversive state enhanced conditioned effects during the CPP testing in Mt mice. Altogether, these data suggest a fast acting negative reinforcement mechanism acting in the Mt mice may be causing a faster establishment of CPP to alcohol, although this hypothesis requires further investigation.

The hypnotic properties of a high dose of alcohol following a single alcohol injection, as measured by the LORR procedure, was not affected by a deficit in αCaMKII autophosphorylation. The hypnotic effects of alcohol are most likely mediated through interaction with $GABA_A$ receptors (Spanagel, 2009), and does not seem not to be under the control of αCaMKII autophosphorylation in the present study. Following subchronic alcohol exposure, all genotype groups were more tolerant to, and recovered sooner from, the hypnotic properties of alcohol to a similar degree. The differential involvement of αCaMKII autophosphorylation in the emotional, rewarding and hypnotic effects of alcohol was not surprising given the multiple mechanisms of action of alcohol at the molecular level. CaMKII is known to play an important role in the plasticity of glutamatergic synapses (Colbran and Brown, 2004; Irvine *et al.*, 2006; Wayman *et al.*, 2008), where as the anxiolytic and hypnotic properties of alcohol are mediated by the GABAergic system, in particular $GABA_A$ receptors (Mehta and Ticku, 1988; Allan *et al.*, 1991). This is

in line with a role for αCaMKII autophosphorylation in the plasticity of glutamatergic, but not GABAergic synapses (Lamsa *et al.*, 2007).

The acute positive reinforcing effects of alcohol are mediated by the recruitment of specific neurotransmitter systems in the reward centres of the brain (Koob *et al.*, 1998a; McBride, 2010). In-vivo microdialysis was used to examine monoaminergic responses which might contribute to the incentive properties, emotional effects and potential arousal regulating effects of alcohol. In agreement with CPP data, alcohol induced an acute sedating effect in Mt mice, an effect which was maintained as alcohol treatments continued. In contrast, alcohol induced a hyperlocomotor response in WT mice. The DA system is critical to experience the acute reinforcing effects of alcohol (Koob, 1992; McBride and Li, 1998; Nestler, 2005), with alcohol typically inducing around a 20% increase in DA levels in the NAcc (Doyon *et al.*, 2003). In the present study, fluid was collected from the NAcc and PFC. Fluid collected from these regions was merely a reflection (a few percent) of the true extracellular concentrations of transmitter present in the brain. Prior to acute alcohol administration, there were no differences in basal DA levels between genotype groups. Microdialysis data showed that an acute alcohol challenge induced a DA increase in the NAcc of WT mice, a response which was entirely absent in Ht and Mt mice. The observation that there are reduced DA levels in the NAcc and reduced drinking behaviours in Mt mice suggests that the diminished DA response may interfere with the experience of the acute rewarding properties of alcohol. This may therefore contribute towards the initially delayed alcohol preference in Mt mice. This study lacks saline control groups, which greatly limits the interpretation of the present data. The design used assumes that the injection procedure affects all genotype groups in the same way (Szumlinski *et al*., 2005; Spanagel *et al*., 2005b). However, based on the findings from Chapter 2 (published in Easton *et al*., 2011), this assumption may be flawed. (Discussed in full in Chapter 6, 4. Strengths and Limitations).

Activation of the dopaminergic neurons in the VTA is believed to be critically involved in the development of addiction (Robbins and Everitt, 1996; Koob *et al.*, 1998b). Projections from the VTA extend to other important

regions implicated in the rewarding potential of drugs of abuse, including the NAcc (Van Bockstaele and Pickel, 1995; Olson *et al.*, 2005), PFC (Carr and Sesack, 2000) and other corticolimbic structures (Albanese and Minciacchi, 1983; Oades and Halliday, 1987). The VTA can be separated into two distinct sub-regions: the rostral and the caudal VTA (Ikemoto *et al.*, 1997b; Ikemoto *et al.*, 1998). These regions contain different neuronal populations (Olson *et al.*, 2005) with more GABAergic neurons present in the rostral VTA as opposed to the caudal sub-region (Olson *et al.*, 2005; Lee *et al.*, 2007; Olson and Nestler, 2007). GABAergic projections originating from the NAcc to the VTA DA neurons represent a potential target for the ethanol modulation of dopaminergic activity (Thielen *et al.*, 2002) and act by disinhibiting VTA DA mediated release (Xiao *et al.*, 2007). Activation of the VTA dopaminergic neurons is one of the cellular bases for ethanol reinforcement (Brodie *et al.*, 1999; Xiao *et al.*, 2009). However, it is the ratio of dopaminergic versus non-dopaminergic neurons in the VTA that has been suggested to contribute to the difference in the functional properties of these sub-regions (Olson and Nestler, 2007). Ethanol has been shown to inhibit GABA release in posterior/caudal DA neurons but facilitate GABA release in the anterior/rostral VTA DA neurons (Guan *et al.*, 2011). Several studies demonstrate that as GABAergic activity in the rostral VTA increases, dopaminergic activity decreases, resulting in a diminished experience of reward and reinforcement. Injections of $GABA_A$ receptor agonists directly into the rostral VTA can decrease the rate of cocaine self-administration in rats (Corrigall *et al.*, 2000). Correspondingly, by blocking $GABA_A$ receptors in anterior/rostral VTA, DA levels increase in the NAcc of rats (Ikemoto *et al.*, 1997a; Ding *et al.*, 2009). Rats have also been shown to self administer $GABA_A$ receptor antagonists into the anterior/rostral VTA (Ikemoto *et al.*, 1997b). In the present study Mt mice showed enhanced c-Fos activation after an acute alcohol injection compared to WT mice in the rostral, but not caudal VTA. This suggests an enhanced GABAergic activity in the rostral VTA, and may be linked to the reduced DA response in the NAcc of the Mt mice. These effects were also observed after an acute alcohol challenge following subchronic administration. As it stands, data were insufficient to be able to directly demonstrate whether

increased cFos expression occurred in the GABAergic neurons. Experiments performing GABAergic neuron and cFos double-labelling would be required in order to more accurately interpret the current cFos data and test the potential contribution of GABAergic neurons to alcohol preference in Mt mice.

Repeated ethanol exposure can induce sensitisation of DRD2 (Perra *et al.*, 2011). As DRD2 is activated $Ca^{2+}$ is released from intracellular stores and CaMKII becomes active (Takeuchi *et al.*, 2002). This activation of CaMKII mediates the calcium dependent desensitisation of DRD2 signalling in DA neurons (Perra *et al.*, 2011) resulting in a loss of receptor function due to continuous agonistic stimulation. This mechanism may be implicated in the diminished DA response seen in the NAcc of WT mice upon subchronic alcohol administration in the present study. Taking past and present data into account, there is strong evidence for the involvement of αCaMKII and αCaMKII autophosphorylation in the adaptation of the DA system in the establishment of alcohol-addiction related behaviors.

It is widely documented that upon alcohol administration, 5-HT activity is increased in the ventral hippocampus (McBride *et al.*, 1993; Virkkunen and Linnoila, 1997; Bare *et al.*, 1998; Thielen *et al.*, 2002). In the current study alcohol did not affect 5-HT activity in the NAcc or PFC in WT or Ht animals. In Mt animals, however, alcohol induced a significant 5-HT increase in the PFC. Deficiencies in brain 5-HT have previously been associated with high ethanol drinking responses (Murphy *et al.*, 1982; McBride *et al.*, 1993; Virkkunen and Linnoila, 1997). Likewise, artificially induced increases in extracellular 5-HT can reduce ethanol drinking (Boyce-Rustay *et al.*, 2006). Alcohol-induced 5-HT increases in the PFC of Mt mice might therefore contribute towards the reduced alcohol drinking behaviours seen in these mice. 5-HT is released in response to alcohol (Daws *et al.*, 2006) and is thought to contribute towards the rewarding effects of alcohol. There is a strong link between CaMKII and serotonergic function in the brain. Tryptophan hydroxylase (TPH) is the rate limiting enzyme in the biosynthesis of 5-HT (Kuhn *et al.*, 2007), and CaMKII is required for the activation of TPH, by phosphorylation (Hamon *et al.*, 1981; Kuhn and Lovenberg, 1982; Ehret *et al.*, 1989). The administration of the

CaMKII inhibitor, KN-62, increases the firing rate of 5-HT neurons (Liu *et al.*, 2005). This finding may be extrapolated to the present data, whereby a reduction in CaMKII function may have caused increased 5-HT firing and therefore transmitter release into the cleft. Microdialysis data showed that alcohol induced 5-HT increases in the PFC of Mt mice. Uncontrollable (inescapable) stressors activate 5-HT neurons in the dorsal raphe nucleus and its projections far more than exposure to escapable stressors (Amat *et al.*, 2005). Taken together, data might suggest that 5-HT increases in the PFC could be due to Mt mice being exposed to an uncontrollable stressor, lending support to the negative reinforcement theory suggested above.

The present study shows that αCaMKII autophosphorylation is important for the normal development of alcohol reinforcement and may act by altering both DA and 5-HT in the meso-corticolimbic system. In light of current findings, one might speculate that alcohol preference is driven by a positive reinforcement mechanism. In Mt mice, both a reduced alcohol preference and a diminished DA response in the NAcc were observed. Alcohol CPP, however, was established at an accelerated rate and may relate to the increased 5-HT levels seen in the PFC of Mt mice in response to alcohol. Previous research suggests that this response may be driven by a negative reinforcement mechanism. Interestingly, Ht mice showed neither a strong DA nor 5-HT response, perhaps suggesting an absence of both positive and negative reinforcement. This was in line with their behaviour since they were the most delayed group in establishing alcohol drinking and alcohol CPP.

NA plays a role in several brain functions including attention and arousal as well as learning and memory (Huether, 1996; Sved *et al.*, 2001). While stimulants have been shown to induce synaptic increases in NA levels (Bardo, 1998; White and Kalivas, 1998), lesions of the noradrenergic system (Roberts *et al.*, 1977) and NA transporter (NET) inhibitors (Wee and Woolverton, 2004; Wee *et al.*, 2006) do not affect cocaine self-administration behaviour. Ethanol consumption has been shown to significantly decrease the rate of NA turnover in specific brain areas such as the NAcc, PFC and amygdala (Smith *et al.*,

2008), and such changes have been hypothesised to contribute to behavioural changes associated with binge drinking (Smith *et al.*, 2008). The present study reported acute noradrenergic increases in the NAcc of WT, but not in Ht or Mt mice, with the effect seen in WT mice normalising over time. The opposite effects were seen in the PFC, with differences in NA levels seen following subchronic, but not acute alcohol treatment. The WT response increased after a further alcohol injection. No such effect was seen in Mt or Ht mice. Basal neurotransmitter levels represent resting DA, 5-HT and NA levels, prior to alcohol treatment, in the NAcc and PFC of alcohol naïve (acute) and alcohol experienced (subchronic) Mt, WT and Ht mice. Data reflected the influence of subchronic alcohol use and the adaptation of the noradrenergic system, and the reward system in general, at baseline between genotype groups. Together, data suggests that alterations in NA transmission may not be critical for, but may contribute to the development of addictive behaviours (Sofuoglu and Sewell, 2009). The observed alcohol-induced NA response may play a part in alcohol drinking.

Both male and female mice were used in all experiments of the present study, and balanced across groups. Sex differences are well known in alcoholism-related behaviors (Desrivières *et al.*, 2011; Lenz *et al.*, 2011), however there were no significant sex differences detected in this study. Data was therefore collapsed for analysis although a subtle sex specific role for αCaMKII in the development of alcohol-related behaviours cannot be ruled out and may be a limitation for the current analyses. The absence of saline injected groups is a major drawback in the present thesis and significantly limits the interpretation of the data. Inclusion of saline groups for each genotype would have helped to identify injection stress-induced increases in behavioural and neurochemical levels, especially since threatening situations and stimuli have been identified as a potential confound (Chapter 2 and Easton *et al.,* 2011). There is a role for DA in processing both rewarding and aversive events (Brischoux *et al*., 2009). It is therefore possible that an aversive stimulus, such as an injection, will increase dopaminergic activity in the NAcc and PFC (Abercrombie *et al*., 1989; Kalivas and Duffy, 1995). Thus, injection stress cannot be ruled out as a

contributing factor and it is not possible to distinguish between responses caused solely by alcohol from those responses caused by the injection procedure. (Discussed in full in Chapter 6, 4. Strengths and Limitations).

Taken together, the present study showed that αCaMKII autophosphorylation is important for the establishment of alcohol related behaviors. αCaMKII autophosphorylation may therefore play a part in the positive reinforcing properties of alcohol mediated by a VTA driven dopaminergic activation of the NAcc. A deficiency in αCaMKII autophosphorylation enhances susceptibility to threatening stimuli, an effect which potentially renders animals vulnerable to the negative reinforcing effects of alcohol. This may be driven by serotonergic activation of the PFC. Taken together, data demonstrate that manipulation of the αCaMKII autophosphorylation mechanism does appear to alter the development of alcohol preference and the rate at which preference is established. This seems to be related to modifications of the dopaminergic and serotonergic systems.

# αCaMKII autophosphorylation contributes to the development of preference but not conditioned hyperactivity or behavioural sensitisation to cocaine.

Chapter 4

## Abstract:

Cocaine is the most commonly used illicit psycho-stimulant drug in the U.K. Addiction to cocaine develops in a considerable number of individuals. It is widely believed that addiction and memory formation share a number of molecular and anatomical pathways. Interestingly, αCaMKII a key mediator of LTP, has been shown to become phosphorylated with chronic cocaine use. The aim of the present study was to investigate whether a deficit in the function of αCaMKII alters preference to cocaine and the rate at which cocaine preference is established. Data indicated a difference in the time-course of the DA increase in the extracellular space of the NAcc following cocaine administration and the pattern of establishment of CPP in αCaMKII Mt mice. 5-HT is also required for the development of CPP and baseline 5-HT levels in the NAcc and PFC were altered in αCaMKII Mt mice. αCaMKII autophosphorylation did not appear to be required for the development of other cocaine-related behaviours associated with chronic administration such as conditioned hyperactivity and behavioural sensitisation. These effects remained stable over time as did noradrenergic levels in the NAcc and PFC. Data generated in the present study suggests that there are other mechanisms at play in the development of addictive states as altered αCaMKII autophosphorylation does not solely account for the development of cocaine preference, but may contribute towards the establishment of cocaine preference.

## 1. Introduction:

20% of the U.S. population using illicit drugs also meet the criteria for dependence (Glantz *et al.,* 2009). Cocaine is the most commonly used illicit psycho-stimulant drug in the U.K. (UNODC, 2011) and develops into addiction in a significant number of individuals. Despite this, the underlying mechanisms associated with addiction remain largely unknown, although it is now widely believed that addiction may be due to dysfunction in the learning and memory pathways (Nestler, 2002a; Kelley, 2004; Hyman, 2005; Müller and Schumann, 2011).

CaMKII activation contributes to the development and maintenance of addictive states (Noda and Nabeshima, 2004; Tang *et al.*, 2006). Chronic cocaine exposure can increase αCaMKII expression levels in the NAcc, which in turn may be essential for the motivation to self administer cocaine (Wang *et al.*, 2010). αCaMKII influences synaptic plasticity and learning and memory processes by interfering with LTP (described in detail in Thesis Chapter 1, section 4.4.1). αCaMKII plays an important role at glutamatergic post-synapses (Fukunaga *et al.*, 1993; Colbran and Brown, 2004; Lengyel *et al.*, 2004; Uys and LaLumiere, 2008) by affecting the trafficking of AMPA glutamate receptors to the synapse (Boehm and Malinow, 2005). Activation of AMPA receptors in the NAcc has been shown to reinstate cocaine seeking (Kalivas *et al.*, 2005; Schmidt *et al.*, 2005), and conversely, AMPA receptor antagonists have been shown to attenuate cocaine reinstatement (Cornish *et al.*, 1999; Park *et al.*, 2002; Suto *et al.*, 2004). Evidence suggests that CaMKII may contribute to the reinstatement of cocaine seeking behaviours. Cocaine is also known to exert its effects by influencing monoaminergic functioning (Ritz *et al.*, 1990) by blockade of the relative transporters, and thereby the re-uptake of monoamines into the pre-synaptic cell (Uhl *et al.*, 2002; Hall *et al.*, 2004; Jones *et al.*, 2009). This causes the synaptic cleft to become flooded with transmitter molecules (Di Chiara and Imperato, 1988; Carboni *et al.*, 1989), resulting in the over stimulation of receptors (Nestler, 2005). This over-stimulation can induce a LTP-like state at the cellular level (Saal *et al.*, 2003; Thomas and Malenka, 2003; Kauer, 2004). Enhancement of synaptic

transmission and lasting potentiation of cell signalling is the major mechanism underlying normal learning and memory formation and is also important for drug memories (Ungless *et al.*, 2001). Disruption of the LTP mechanism, and consequently of learning and memory, may therefore have the potential to affect the establishment of drug addiction.

αCaMKII has been identified as a link between the molecular pathways of addiction (Li *et al.*, 2008), a theory which is supported by the important contributions of the glutamatergic and dopaminergic systems in the development of cocaine-related behaviours (Anderson *et al.*, 2008). Anderson and colleagues (2008) report that cocaine reinstatement (following cocaine self-administration and extinction phases) is successively associated with the enhancement of; DRD1-like (DRD1 and DRD5) receptor activation, L-type voltage-gated $Ca^{2+}$ channel stimulation, $Ca^{2+}$ influx through L-type channels, subsequent CaMKII activation, phosphorylation of CaMKII at Thr286, glutamate receptor 1 (GluR1) subunit containing AMPA receptors on Ser831 (a CaMKII phosphorylation site), and cell surface expression of GluR1 containing AMPA receptors. In concordance with these findings, impairment of GluR1 trafficking to the cell surface was found to attenuate cocaine-induced reinstatement behaviour. There is strong evidence for a link between the dopaminergic and glutamatergic transmitter systems, more specifically between cocaine-induced DRD1 activation, downstream CaMKII activation and AMPA trafficking (Anderson *et al.*, 2008). Interestingly, the phosphorylation site on CaMKII (Thr286) mentioned in the Anderson (2008) study is also the site of the targeted mutation in the mice used in the present study.

Psychostimulants typically increase extracellular DA in the NAcc and striatum (Kalivas and Stewart, 1991; Robinson and Berridge, 1993; Pierce and Kalivas, 1997b). L- or N-type $Ca^{2+}$ channel antagonism or inhibition of CaMKII blocks amphetamine-induced DA release into the NAcc (Pierce and Kalivas, 1997a). L-type $Ca^{2+}$ channel antagonism also blocked cocaine-induced increases in extracellular DA in the neostriatum of cocaine sensitised rats (Pani *et al.*, 1990). A growing body of research indicates that $Ca^{2+}$ and $Ca^{2+}$ mediated second messenger systems influence behavioural sensitisation

to psychostimulants via the elimination of this DA increase (Pierce *et al*., 1998). Specifically, CaMKII inhibition impairs the behavioural expression of sensitization to cocaine (Pierce *et al*., 1998).

There is an increasing amount of literature implicating αCaMKII in the development of addiction. Continuing on from the investigation into the role of αCaMKII in the establishment of alcoholism-related behaviors (Thesis Chapter 3), the effect of αCaMKII on preference and response to a drug from a different class, cocaine, was undertaken. The main hypothesis proposes that since αCaMKII autophosphorylation deficiency has previously been shown to modify alcohol addiction behaviours (Thesis Chapter 3), this deficit will also alter the speed at which cocaine preference is established. In order to further evaluate the development of addictive behaviours, the establishment of cocaine-induced conditioned place preference was assessed. Since cocaine exerts its effects by influencing monoaminergic functioning (Ritz *et al.*, 1990), the acute positive reinforcing effects of cocaine was also monitored using in vivo microdialysis performed in brain regions associated with drug reward. Regions to be dialysed (NAcc and PFC) were selected based on the degree of overlap between the regions in which DA and other monoamines are released in response to cocaine administration, and regions in which αCaMKII is abundantly expressed. This selection was based on the previously mentioned criteria, and that the PFC receives input from projections of the NAcc.

Changes in monoaminergic functioning may modulate the incentive properties, emotional effects and potential arousal regulating effects of cocaine. The current study aims to establish whether the motivational effects of cocaine are influence by the autophosphorylation of αCaMKII.

## 2. Materials and Methods:

All housing and experimental procedures were performed in accordance with the U.K. Home Office Animals (Experimental Procedures) Act 1986.

### 2.1 Animals

Male and female αCaMKII$^{T286A}$ mice (Giese *et al.*, 1998) were studied in sex balanced designs in all experiments (for details of the genetic background: see Chapter 2, Materials and Methods; section 2.1). This mutation blocks the autophosphorylation of CaMKII but does not affect the $Ca^{2+}$-dependent activity (Giese *et al.*, 1998). Animals were individually housed in Tecniplast cages (32cm x 16cm x 14cm), using Litaspen sawdust and nesting materials, (Sizzlenest, Datsand, Manchester UK). Mice were provided with food and water ad libitum, and kept on a 12:12 hour light: dark cycle (lights on at 7.00 am). Behavioral tests were performed during the light cycle between 09:00 and 16:00 h. Room temperature was maintained between 19℃ and 22℃ at a humidity of 55% (±10%).

### 2.2 Conditioned place preference (CPP)

*2.2.1 Apparatus*

For details see Chapter 3, Materials and Methods section 2.5.1.

*2.2.2 CPP Establishment*

Cocaine naïve animals (Mt: n=14; WT: n=14; Ht: n=16) were injected (i.p.) immediately before each trial with either saline, or cocaine solution at a dose of 20mg/kg. Mice were then instantly transferred to the testing suite and placed into the CPP boxes, signifying the beginning of the trial period (see Chapter 3, Materials and Methods section 2.5.2. Fig. 3.2A). The experiment involved four phases (Table 4.1); habituation trial (one session), conditioning trials (14 sessions), preference tests (5 sessions) and an incubation period (6 days). Trials were performed once daily. *Habituation (day 1):* The intention of

the habituation session was to acclimatise mice to the test procedure and apparatus prior to commencing the experiment. Mice were injected with saline and introduced into the centre compartment with free access to all three compartments for 20 minutes. *Pre-test (day 2):* The pre-test was designed to establish a baseline level of preference for each individual animal. Mice were conditioned to either their preferred or non preferred compartment using a counterbalanced experimental design. Mice were injected with saline and introduced into the centre compartment with free access to all three compartments for 20 minutes. *Conditioning trials (days 3-4, 6-9 and 11-18):* Conditioning trials were performed in pairs, odd numbered pairings were conditioned with cocaine, and even numbered pairings were conditioned with saline, this was balanced across groups. All animals received 7 pairings with saline and 7 pairings with cocaine in total. Mice were injected with either saline or a cocaine solution (20mg/kg i.p.) and introduced into one of two compartments, with restricted access, for 20 minutes. *Preference tests (days 5, 10, 19 and 26):* In order to monitor the time course of CPP establishment, preference tests were systematically dispersed between conditioning trials on days 5, 10, 19 and 26. Thus, preference tests were performed after 1 pair of conditioning trials (consisting of 1 cocaine pairing and 1 saline pairing), after 3 pairings and again after 7 pairings (represented in the figures). Before each test, mice were injected with saline and introduced into the centre compartment with free access to all three compartments for 20 minutes. *Incubation period (days 20-25):* In order to test the consolidation of CPP, animals were left undisturbed in their home cages on days 20-25, and then re-tested for CPP.

**Table 4.1:** The entire experimental protocol each mouse was exposed to during the conditioned place preference procedure

| Trial | Duration | Treatment |
|---|---|---|
| **Habituation** | **5 mins** | **Saline** |
| **Pre Test** | **20 mins** | **Saline** |
| **Conditioning x 2** | **20 mins** | **Cocaine/Saline** |
| **Preference Test 1** | **20 mins** | **Saline** |
| **Conditioning x 4** | **20 mins** | **Cocaine/Saline** |
| **Preference Test 2** | **20 mins** | **Saline** |
| **Conditioning x 8** | **20 mins** | **Cocaine/Saline** |
| **Preference Test 3** | **20 mins** | **Saline** |
| **Incubation** | **6 days** | **None** |
| **Preference Test 4** | **20 mins** | **Saline** |

### 2.3 In-vivo Microdialysis

In order to test whether the lack of αCaMKII autophosphorylation would lead to altered monoaminergic responses to cocaine treatment, in vivo microdialysis was performed and dopaminergic, serotonergic and noradrenergic levels measured. Cocaine influences monoaminergic functioning (Ritz et al., 1990) by blocking the relative transporters, and thereby the re-uptake of monoamines into the pre-synaptic cell (Uhl et al., 2002; Hall et al., 2004; Jones et al., 2009). Transmitter flooding of the synaptic cleft (Di Chiara and Imperato, 1988; Carboni et al., 1989), results in the over stimulation of receptors (Nestler, 2005) and can induce a LTP-like state at the cellular level (Saal et al., 2003; Thomas and Malenka, 2003; Kauer, 2004). It is this enhancement of synaptic transmission and lasting potentiation of cellular signalling which underlies normal learning and memory formation, and is also important for the formation of drug-related memories (Ungless et al., 2001). Activation of AMPA receptors in the NAcc can reinstate cocaine seeking (Kalivas et al., 2005; Schmidt et al., 2005) whereas antagonism of these receptors can attenuate cocaine reinstatement (Cornish et al., 1999;

Park et al., 2002; Suto et al., 2004). Although assessment of glutamate levels in the present study was clearly warranted, it was not possible to measure glutamate alongside the other transmitters of interest as an HPLC set-up with UV detection would have been required. As such, an HPLC system was not available, priority was given to the measure of extracellular DA, 5-HT and NA. Due to the high technical demand of dialysis, a strongly focused design was used, according to Spanagel *et al.,* (2005b) and Szumlinski *et al*., (2005).

*2.3.1 Surgery*

For details see Chapter 3, Materials and Methods section 2.7.1.

*2.3.2 Procedure*

For details see Chapter 3, Materials and Methods section 2.7.2.

*2.3.3 Acute cocaine effects*

Cocaine naïve animals were used for this test (Mt: n=8; WT: n=8; Ht: n=8). In-vivo microdialysis was performed, during which animals received a single injection of cocaine (20mg/kg, i.p.). This provided an acute neurochemical response to cocaine treatment. Once microdialysis experiments were complete, animals were sacrificed by cervical dislocation. Brains were fixed in 4% formaldehyde solution and stored at 4 ℃. Brains were sliced on a microtome and stained with cresyl violet for analysis of probe placement. Since all probes were surgically inserted accurately within the NAcc and PFC regions, all animals were considered for data analysis. For details see Chapter 3, Materials and Methods section 2.7.3. Fig. 3.4 & 3.5.

*2.3.4 HPLC-ED analysis*

All samples were analysed using HPLC-ED to measure DA, 5-HT and NA levels in response to cocaine administration. For details see Chapter 3, Materials and Methods section 2.7.4.

## 2.4 Statistical Analysis

All graphical output data is expressed as a mean ± SEM. *CPP:* place preference data were initially log transformed for the analysis presented in Fig. 4.1 only. The logarithmic transformation is widely used to normalise skewed data (Sedgwick, 2012; Feng *et al*., 2013). This is a valuable method since it makes data more interpretable and helps to meet assumptions of statistical tests. The main advantage of this is that it permits the use of parametric tests as opposed to non-parametric ones (Sedgwick, 2012). A logarithmic transformation is typically used to stabilise the variance of a sample if it was not already present (Bland, 2000; Sedgwick, 2012), and was the reason for the data transformation. All data were analysed using one-way or two-way ANOVA followed by planned pairwise comparisons Fisher's LSD tests or Bonferroni corrected t-tests. *Microdialysis:* Baseline neurochemical data were analysed using one way ANOVA and planned Fisher's LSD tests. Cocaine induced neurochemical effects were expressed as a percentage of the mean of the three baseline samples which were taken as 100% in an attempt to standardise the data across groups. Data were compared using two-way ANOVA with factors genotype (3) and time (12). To compare cocaine effects of certain time points, planned pairwise comparisons were performed using Fisher's LSD tests. One way repeated measures ANOVA and post hoc Fisher LSD tests were performed and used to determine differences between pairs of values within groups. Sex differences have been well established in clinical and rodent studies of cocaine-related behaviors (Festa and Quinones-Jenab, 2004) but we did not see significant sex differences in this study. Therefore, data were collapsed for analysis. The software SPSS 17.0 and Statistica 9 were used. A significance level of $p < 0.05$ was used to test for statistical significance.

As discussed in Chapter 2, Section 2.3. Statistical Analysis, the present study retrospectively used the 'Resource Equation' (Mead, 1988) method to quantify the probability that experiments detected biologically important effects. Mead's resource equation (not using blocking) states:

**E = (total number of experimental units) – (number of treatment combinations)**

**E** was calculated for all experiments in the present study. CPP, **E** = 28; MD, **E** = 18; According to Mead's rule, **E** should be between 10 and 20. If **E** is less than 10, increasing numbers would lead to good returns. If **E** is over 20, resources may be wasted. As reasoned in Chapter 3, Section 2.9. Statistical Analysis, there are circumstances where it is justifiable for **E** to be greater than 20. Such as, when experiments are complex and involve several factors e.g. both sexes, different time points and more than one genotype.

# 3. Results

## 3.1. Establishment of cocaine place preference is delayed in αCaMKII deficient mice

The place preference data presented in Fig. 4.1 were log transformed to stabilise the variance in the sample (Bland, 2000; Sedgwick, 2012). Two-way ANOVA for CPP establishment (test trials 1-3) revealed a significant effect of the genotype ($F_{2,41}$=3.64, p=0.035) and test trial ($F_{2,82}$=3.26, p=0.043), but no significant interaction (p>0.05; Fig. 4.1). After a single cocaine pairing, Mt mice showed a reduced preference for the cocaine-paired compartment compared to WT (t=2.19, p=0.002) animals. After three cocaine pairings, preference increases in all groups and WT preference is significantly increased compare to Mt (t=2.29, p=0.0002) but not Ht animals (p>0.05). After seven cocaine pairings preference slightly diminishes in all genotype groups, but WT mice still exhibit a significantly higher CPP than Mt (t=2.08, p<0.0001) and Ht (t=2.13, p<0.0001) mice. After a seven day period with no cocaine treatment or conditioning, Mt mice showed the same level of preference as Ht and WT mice (one-way ANOVA, p>0.05).

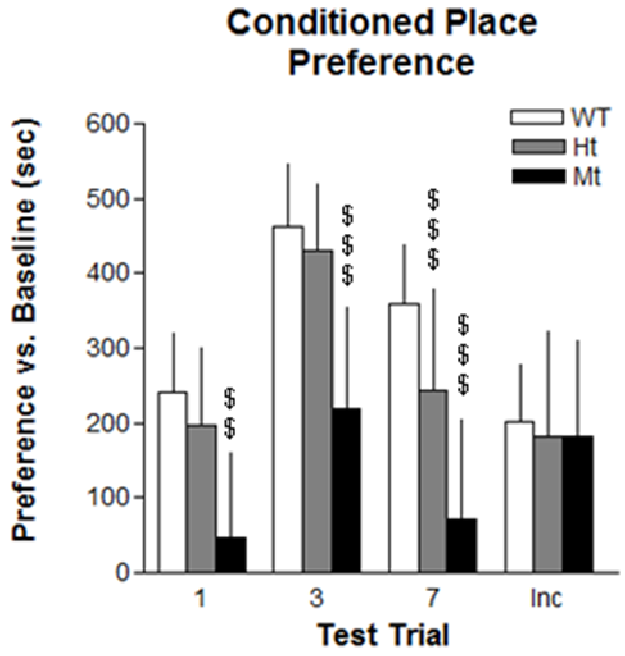



**Figure 4.1:** Conditioned place preference (CPP) is impaired in αCaMKII autophosphorylation deficient mice. Preference vs. baseline (sec.) in the conditioning (cocaine paired) compartment during a 20 minute test trial. Planned pairwise comparisons, T-test, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs. WT.

### 3.2. Conditioned cocaine-induced hyperactivity is not altered by genotype

Two-way ANOVA revealed a significant time effect ($F_{4,152}=10.60$, $p<0.0001$) of cocaine-induced hyperactivity in the cocaine-paired compartment. There was a significant conditioned hyperlocomotion effect seen in all genotype groups in the cocaine compartment (Fig 4.2A; BL vs. trial 1: t= -2.45, p=0.019; BL vs. trial 3: t= -5.55, p<0.0001; BL vs. trial 7: t= -3.72, p=0.001; BL vs. INC: t= -3.68, p=0.001) but not in the saline compartment (Fig 4.2B; All, p>0.05) compared to baseline. This suggests that the αCaMKII autophosphorylation mechanism does not influence conditional hyperactivity, a type of behaviour associated with cocaine administration.

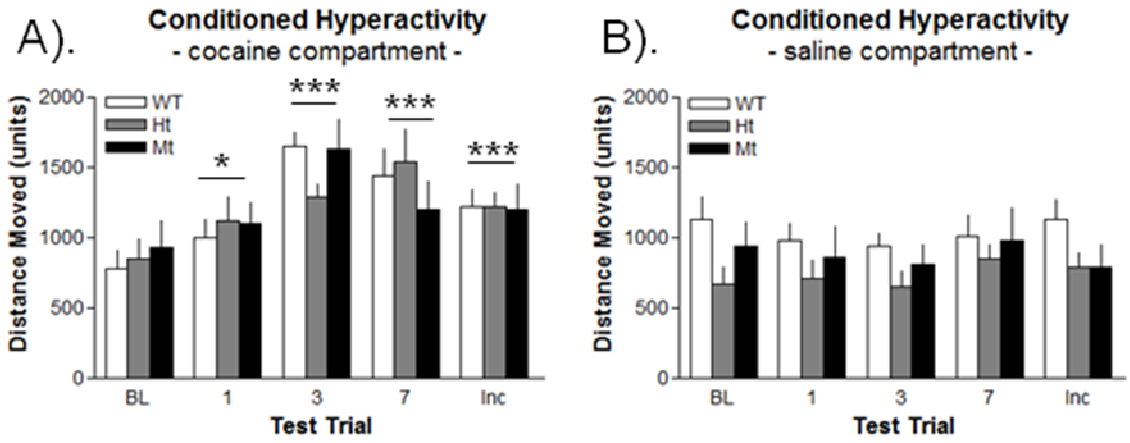



**Figure 4.2:** αCaMKII autophosphorylation does not affect conditioned hyperactivity to cocaine. Distance moved (units) in the conditioning (cocaine paired) compartment (A) and the pseudo-conditioning (saline paired) compartment (B) during a 20 minute test trial. t-test for test trial vs. baseline (BL) *p<0.05, **p<0.01, ***p<0.001.

### 3.3. Sensitisation effects of cocaine are not altered by genotype

The acute locomotor responses to cocaine (Fig 4.3A) and saline (Fig 4.3B) were identical in all groups (p>0.05) and do not appear to be affected by the absence of αCaMKII autophosphorylation. Two-way ANOVA revealed a significant time effect ($F_{1,42}$=28.25, p<0.0001), but no genotype effect or interaction (p>0.05). Planned pairwise comparisons confirmed a significant increase in activity after seven cocaine pairings compared to activity levels after only one pairing (Fig 4.3A; WT: t= -2.92, p=0.011; Ht: t= -3.47, p=0.003; Mt: t= -2.85, p=0.014). This effect was not seen in the saline-paired compartment (Fig 4.3B; p>0.05). Data show that cocaine produces considerable behavioural stimulating effects in all genotypes which sensitises over seven treatments. This typre of behaviour does not seem to require αCaMKII autophosphorylation.

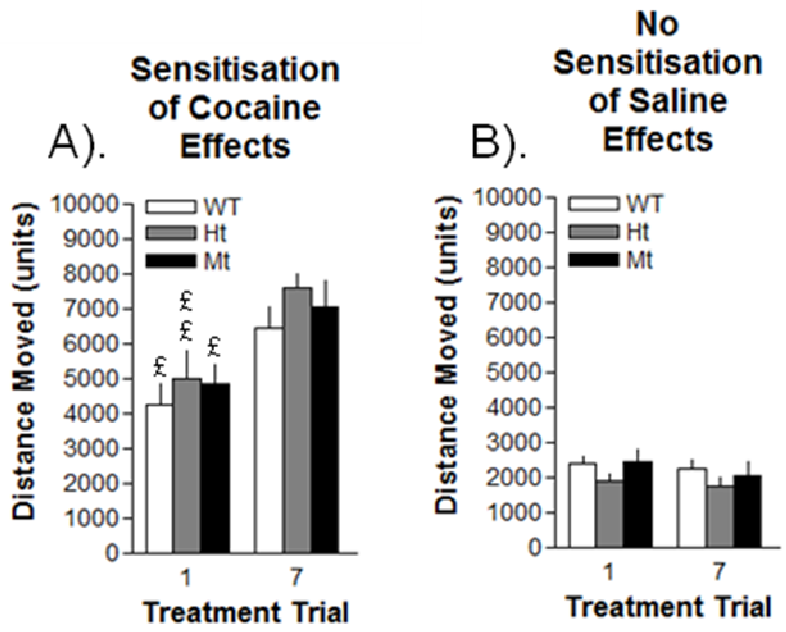



**Figure 4.3:** αCaMKII autophosphorylation is not required for the conditioned behavioural sensitisation effects of cocaine. Distance moved (units) in the conditioning (cocaine paired) compartment (A) and the pseudo-conditioning (saline paired) compartment (B) during a 20 minute test trial after one and seven pairings. t-test for trial 7 vs. trial 1 $^{£}p<0.05$, $^{££}p<0.01$.

### 3.4. Basal transmitter levels may explain blunted monoamine responses to cocaine administration

DA levels in the NAcc were comparable between WT and Mt mice, but significantly elevated in Ht mice ($F_{2,55}$= 16.25, p<0.001; LSD: vs. WT, p<0.001; vs. Mt, p<0.001; Fig. 4.4A). DA levels in the PFC (Fig. 4.4B) were significantly greater in Ht and Mt mice compared to WT levels ($F_{2,60}$= 4.13, p=0.02; LSD: vs. Ht, p=0.03; vs. Mt, p=0.01). Baseline 5-HT levels were elevated in the NAcc (Fig. 4.4C) of Ht and Mt mice compared to WT ($F_{2,59}$= 5.43, p=0.007; LSD vs. Ht, p=0.006; vs. Mt, p=0.004). Levels of 5-HT in the PFC (Fig. 4.4D) were greater in the Mt compared to both WT and Ht mice ($F_{2,64}$= 10.45, p<0.001; LSD: vs. WT, p=0.003; vs. Ht, p=<0.001). NA levels in the NAcc (Fig. 4.4E) were comparable between Mt and WT mice, although levels of NA in Ht mice were elevated compared to other groups ($F_{2,58}$= 4.25,

p=0.02; LSD: vs. WT, p=0.006 ; vs. Mt, p=0.05). Basal NA levels in the PFC (Fig 4.4F) were significantly lower in Mt mice ($F_{2,61}$= 5.31, p=0.007; LSD: vs. WT, p=0.006; vs. Ht, p=0.006).

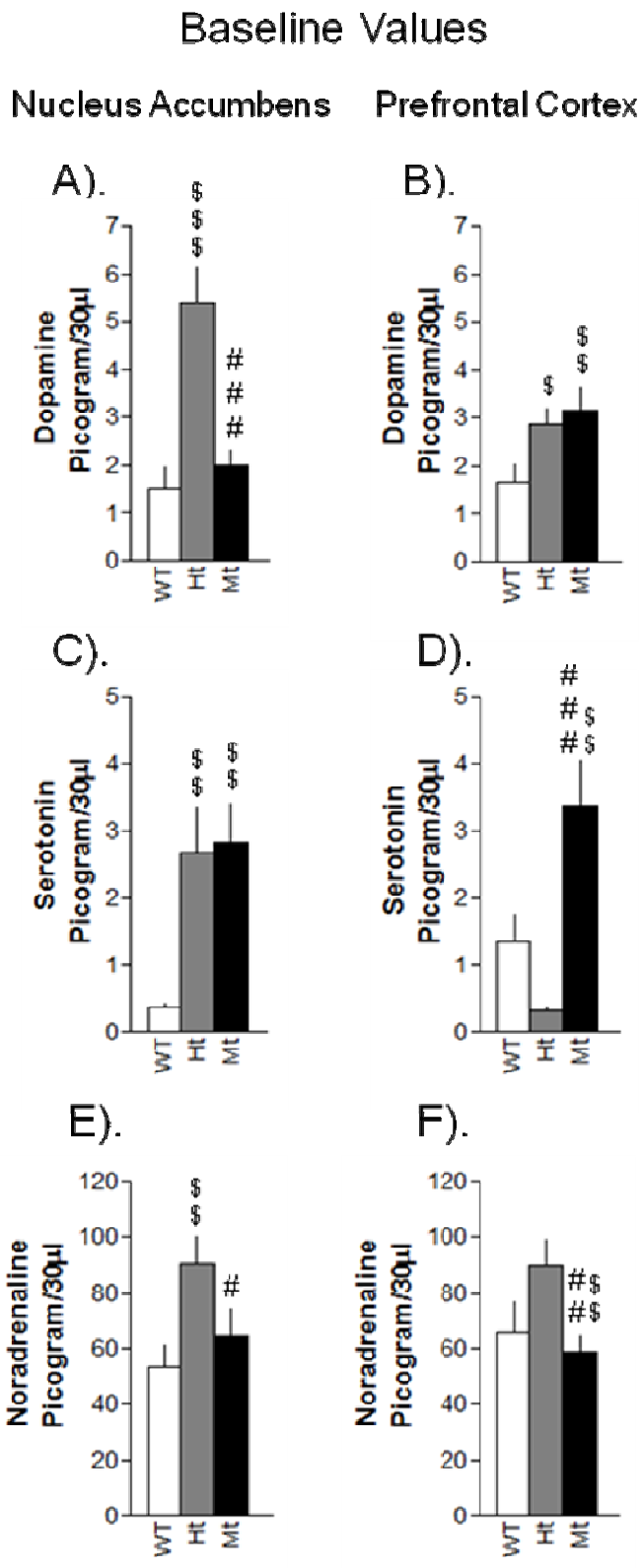



**Figure 4.4:** Baseline monoamine levels. Bars represent Neurotransmitter levels in the NAcc prior to cocaine treatment for DA (A); 5-HT (C); and NA (E). Baseline Neurotransmitter levels in the PFC prior to cocaine treatment for DA (B); 5-HT (D);

and NA (F). Fisher LSD; $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs. WT; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. Ht.

### 3.5. Cocaine alters extracellular cocaine-induced DA levels in the NAcc of αCaMKII autophosphorylation deficient mice

Two-way ANOVA revealed significant effects of time and genotype (time: $F_{11,165}= 2.04$, p=0.03; genotype: $F_{2,15}= 6.41$, p=0.01) and a significant interaction (time x genotype: $F_{22,165}= 2.12$, p=0.004; Fig. 4.5). Post hoc LSD analysis reveals a difference between Mt and Ht mice (LSD: Ht vs. Mt, 40mins p=0.013, 80mins p=0.010, 100mins p=0.011, 120mins p=0.003, 140mins p=0.032 after alcohol injection; Fig. 4.5). Data suggest that after cocaine administration, WT mice showed a tendency towards an increased DA response in the NAcc, which lasted for 40 minutes. The DA response in the Mt group did not show the same activation pattern and slowly climbed during the test. The Ht DA response in the NAcc remained at basal levels throughout (p>0.05).

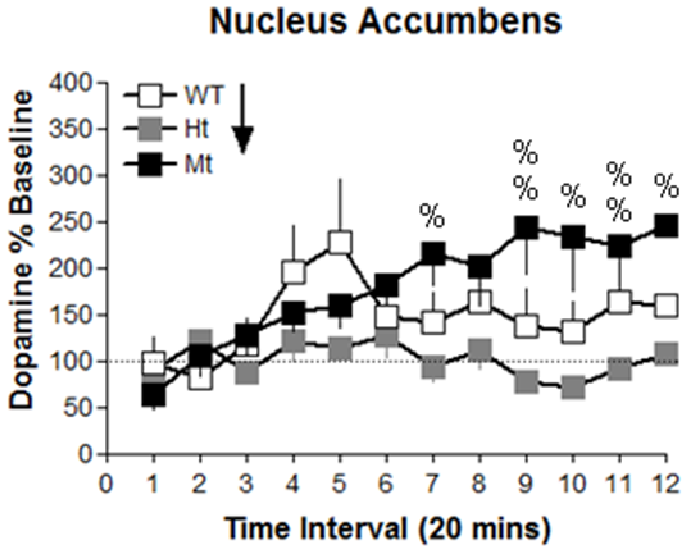



**Figure 4.5:** Speed of initiation of dopamine (DA) peak response in the nucleus accumbens (NAcc) may account for the difference in the development of cocaine-induced conditioned place preference (CPP) between αCaMKII autophosphorylation deficient and WT mice. Extracellular DA levels above baseline in the NAcc after

cocaine treatment. Each point represents a 20 minute time window. Arrow indicates time of cocaine treatment. Planned pairwise comparisons using Fisher's LSD test between groups at each time point, $^{\%}p<0.05$, $^{\%\%}p<0.01$ Mt vs. Ht.

### 3.6. DA levels following an acute injection of cocaine in the PFC are significantly blunted in αCaMKII autophosphorylation deficient mice

There was an increase in extracellular DA following cocaine treatment (two-way ANOVA; time: $F_{11,110}= 8.61$, $p<0.001$; Fig. 4.6) which peaked 40 minutes after cocaine injection in all groups.This increase in extracellular DA was diminished in the PFC in αCaMKII autophosphorylation deficient mice after a cocaine injection (Fig. 4.6). There was an effect of genotype (genotype: $F_{2,10}= 4.42$, $p=0.04$; LSD: WT vs. Ht, 60mins $p=0.015$ after alcohol injection) since the DA response was diminished in Mt and Ht mice compared to WT.

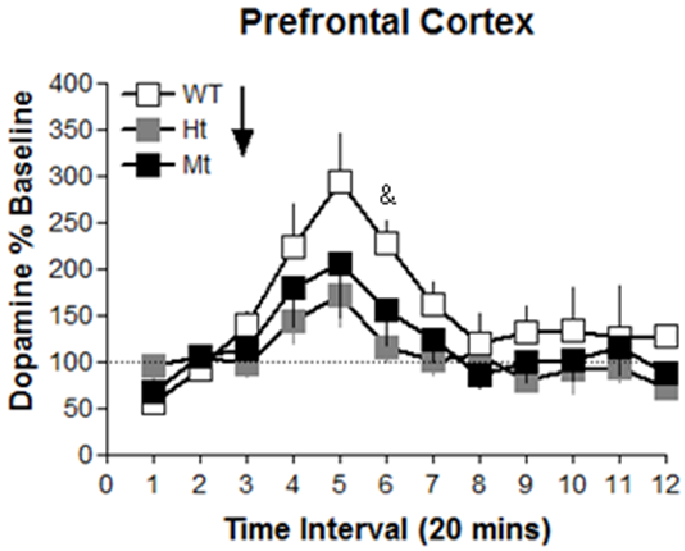



**Figure 4.6:** Dopamine (DA) response in the prefrontal cortex (PFC) is diminished in αCaMKII autophosphorylation deficient mice compared to WT animals after cocaine administration, and may contribute towards the speed of establishment of cocaine-induced CPP. Extracellular DA levels above baseline in the PFC after cocaine treatment. Each point represents a 20 minute time window. Arrow indicates cocaine treatment. Planned pairwise comparisons using Fisher's LSD test between groups at each time point, $^{\&}p<0.05$ WT vs. Ht.

### 3.7. 5-HT response to cocaine administration is not altered in αCaMKII$^{T286A}$ mice

5-HT levels were increased in response to cocaine in the NAcc (two-way ANOVA; time: $F_{11,176}$= 1.99, p = 0.032; LSD, 20mins p=0.05 after alcohol injection WT vs. Ht; Fig. 4.7A). Although, there was no effect of genotype (genotype: $F_{2,16}$= 1.49, p = 0.25). In the PFC there were no significant effects revealed by two-way ANOVA, although exploratory post hoc analysis indicated a genotype difference 60mins post alcohol injection (LSD: p=0.012 Mt vs. WT, p=0.05 WT vs. Ht; Fig. 4.7B).

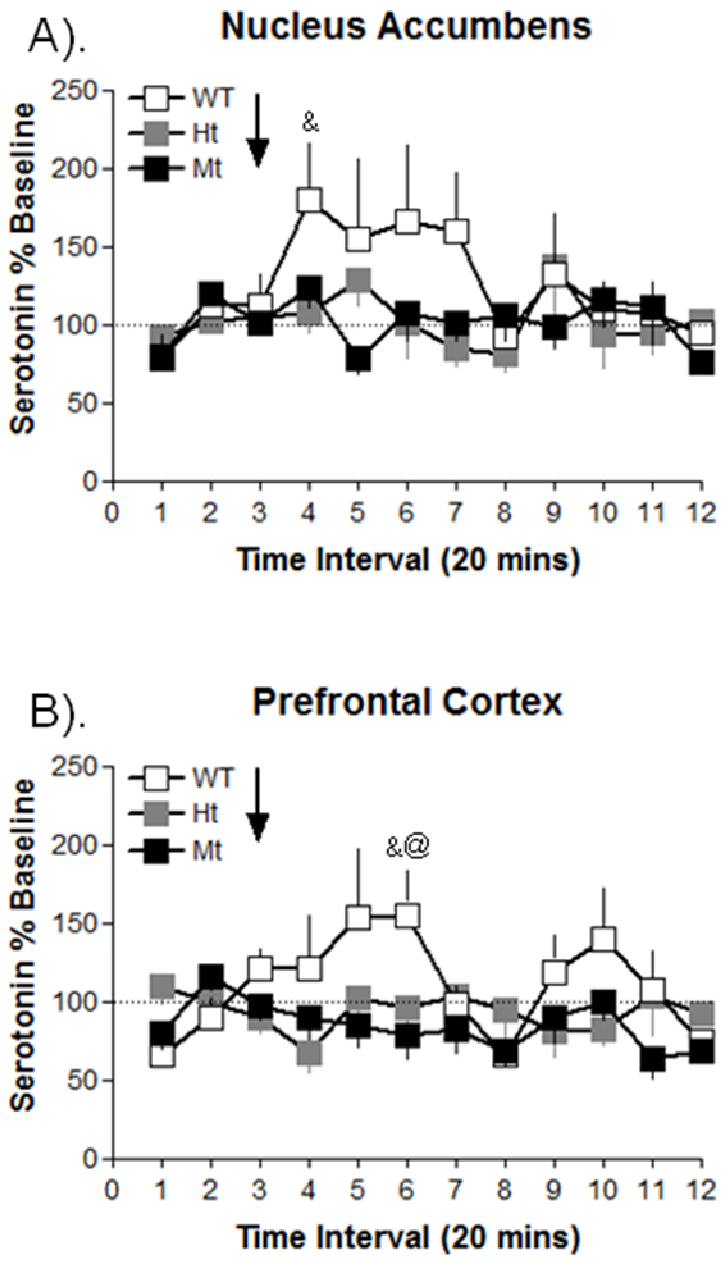



**Figure 4.7:** Serotonin (5-HT) response in the nucleus accumbens (NAcc) and prefrontal cortex (PFC) is absent in αCaMKII autophosphorylation deficient mice after cocaine administration, and may contribute towards the delayed establishment of cocaine-induced conditioned place preference (CPP). Extracellular 5-HT levels above baseline in the NAcc (A) and PFC (B) after cocaine treatment. Each point represents a 20 minute time window. Arrow indicates time of cocaine treatment. Planned pairwise comparisons using Fisher's LSD test between groups at each time point, $^{@}p<0.05$ WT vs. Mt; $^{\&}p<0.05$ WT vs. Ht.

### 3.8. αCaMKII autophosphorylation is not required for cocaine-induced NA increases in the NAcc and PFC

NA levels in the NAcc increased in all genotype groups after cocaine treatment (time: $F_{11,176}$= 14.5, p<0.001; Fig. 4.8A). This response was also seen in the PFC (time: $F_{11,187}$= 10.62, p<0.001; LSD; 20mins after alcohol p=0.012 WT vs. Ht; Fig. 4.8B) and unaffected by αCaMKII genotype.

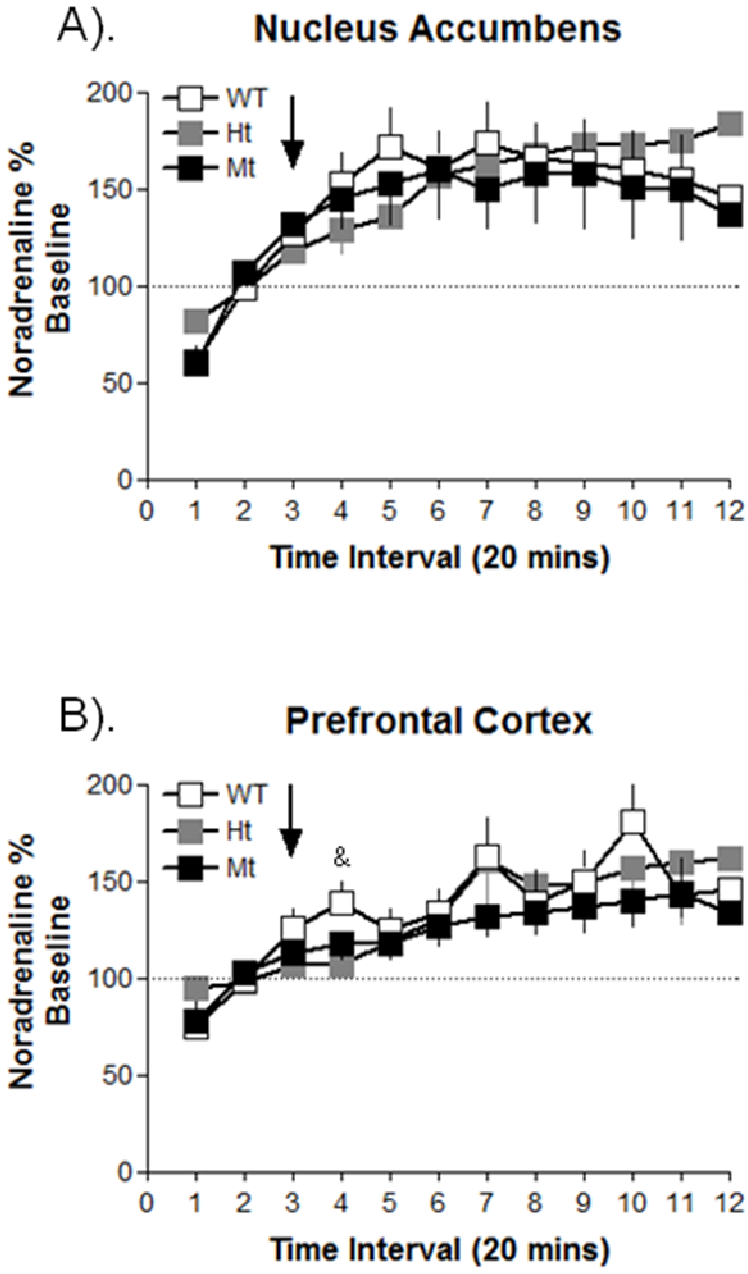



**Figure 4.8:** Noradrenaline (NA) response in the nucleus accumbens (NAcc) and prefrontal cortex (PFC) is comparable between genotype groups after cocaine administration, and may account for the observed behavioural stimulating effects of cocaine. Extracellular NA levels in the NAcc (A) and PFC (B) after cocaine treatment. Each point represents a 20 minute time window. Arrow indicates cocaine treatment. Planned pairwise comparisons using Fisher's LSD test between groups at each time point, $^{\&}p<0.05$ WT vs. Ht.

## 4. Discussion

The activation of CaMKII has been shown to contribute to the development of addictive states (Noda and Nabeshima, 2004; Tang et al., 2006). Increased αCaMKII expression levels in the NAcc has been reported following chronic cocaine exposure and thought to be essential for cocaine self-administration behaviour (Wang et al., 2010). Given the evidence for a connection between αCaMKII autophosphorylation and alcohol-related behaviours (Chapter 3), the specific role of αCaMKII autophosphorylation in the development of cocaine preference was investigated in the present study. αCaMKII autophosphorylation deficiency causes learning impairments (Irvine et al., 2005), but does not alter the capacity to learn, long term memory storage or memory retrieval (Irvine et al., 2006). This suggests that autophosphorylation may affect the speed of normal learning (Lengyel et al., 2004; Lee et al., 2009). Therefore, the hypothesis tested was that the behavioural and motivational properties associated with cocaine use would initially be attenuated in Mt mice, and the effect should normalise to WT levels after repeated treatments. Furthermore, the initial delay should coincide with changes in the monoaminergic system.

Data suggest that αCaMKII autophosphorylation deficient (Mt) mice were initially impaired in their ability to develop cocaine-induced CPP compared to Ht and WT mice. Cocaine can induce a robust CPP (Seale and Carney, 1991; Cunningham *et al.*, 1999), and the typical CPP acquisition pattern was established in WT mice in the present study. Mt mice developed CPP after repeated cocaine treatments and an incubation period of 6 days, during which CPP was consolidated. Cocaine's effects may not be experienced similarly by all genotypes and may have caused the altered development of CPP in αCaMKII Mt mice. Conditioned hyperactivity, as measured in the CPP test design, was not affected by genotype. The acute locomotor and conditioned behavioural sensitisation effects of cocaine were also comparable across genotype groups. Data indicate that the establishment of these behaviours may not require the specific involvement of the αCaMKII autophosphorylation mechanism. There were no cocaine-induced behavioural differences

contributing to the establishment of CPP in αCaMKII mice. Therefore, microdialysis experiments focused on whether alterations in the reward system could be responsible for the establishment of cocaine preference. Resting monoamine levels and changes in these transmitters in response to a cocaine injection were measured in the mesolimbic system since the rewarding and motivational properties of cocaine may be experienced differently by αCaMKII genotype groups and may contribute to the development of cocaine preference.

Cocaine induced DA increases in the NAcc of WT and Mt, but not Ht mice. Data showed a statistically significant difference between Mt and Ht groups only. WT mice showed a tendency towards an increased DA response in the NAcc immediately after cocaine injection. The same activation pattern was not seen in Mt mice in which DA response increased slowly over the three hours following cocaine administration.

It is widely believed that the faster the delivery of a drug with addictive potential into the reward system of the brain, the greater the susceptibility to addiction (Gossop *et al.*, 1992; Hatsukami and Fischman, 1996; Winger *et al.*, 2004). It has been suggested that rapid administration of drugs increases the addictive potential because they produce greater euphoric effects (de Wit *et al.*, 1993; Hatsukami and Fischman, 1996). Drugs are capable of reorganising brain regions implicated in reward and motivation (Samaha and Robinson, 2005). Repeated drug exposure can therefore cause drugs and drug-cues to become increasingly able to control successive behaviours (Robinson and Berridge, 1993; Robinson and Berridge, 2003). Samaha and Robinson (2005) conclude that neuroadaptive processes are clearly initiated and altered by drugs of abuse, and are sensitive to the rate of drug administration. The authors suggest that rate of administration will therefore facilitate neurobehavioural plasticity, which in turn contributes to excessive drug use. The tendency for immediate cocaine-induced increase in extracellular DA in the NAcc of WT mice in the present study may contribute towards the enhanced development of CPP. The slower initiation of this response in Mt mice could therefore account for the delay in cocaine-induced CPP. Results in

the present study are based on genotype comparisons rather than within group comparisons to baseline DA levels. Saline injected groups are also missing from this study and as a result the identification of cocaine specific response cannot be fully determined. Data should therefore be interpreted with caution .

The DA response in the PFC was blunted in Mt compared to WT mice although this difference was not statistically significant. Activation of the VTA DA system, particularly within the shell sub-region of the NAcc, plays a key role in brain reward mechanisms and mediates the behaviour measured in the CPP task (McBride *et al.*, 1999). The VTA projects into several important regions implicated in the motivationally rewarding properties of drugs of abuse including the NAcc (Van Bockstaele and Pickel, 1995; Olson *et al.*, 2005) and the PFC (Carr and Sesack, 2000). While DA is important for the establishment of CPP, it is not sufficient to produce preference, as demonstrated using DAT KO mice which retain cocaine-induced CPP (Sora et al., 1998). DA impairment in the PFC may therefore contribute towards, but cannot solely account for, the impaired development of cocaine-induced CPP.

5-HT is a major contributor in the establishment of cocaine preference and based on research using 5-HT transporter (SERT) KO mice, 5-HT also has been shown to contribute to the development of CPP (Sora *et al.,* 1998; Hall *et al.,* 2002; Hall *et al.,* 2009). Depletion of 5-HT levels in the medial PFC also significantly reduces cocaine-induced CPP (Pum *et al.*, 2008a). Present data showed that cocaine did not induce any robust 5-HT increases in the mesolimbic system. WT mice did show a tendency towards cocaine-induced 5-HT increases in both the NAcc and PFC of WT mice, but this effect was not seen in Ht or Mt mice.

Cocaine induces dose-dependent increases in 5-HT levels in the PFC (Pum *et al.*, 2007) and the NAcc (Müller *et al.*, 2002). A reduction of 90% in 5-HT in the PFC significantly reduces cocaine-induced CPP and also attenuates cocaine-induced hyperlocomotion (Pum *et al.*, 2008a). In the present study, WT mice developed CPP at a typical rate and also showed a mild trend for cocaine-induced increases in 5-HT levels in the NAcc and PFC. Absence of a

cocaine-induced 5-HT response in Mt mice may have contributed towards the observed CPP deficit. The response of Ht mice to cocaine administration was rather ambiguous since the mice developed CPP at the same rate as WT mice, but had no cocaine-induced 5-HT response in either brain region. CaMKII activation has been reported to play a key role in the development and maintenance of an addictive state (Li *et al.*, 2008). αCaMKII is robustly induced by chronic cocaine use and is required for the maintenance of motivation for cocaine (Wang *et al.*, 2010), highlighting αCaMKII as a key molecule in the regulation of cocaine reward. Taking WT and Ht data together, one may theorize that αCaMKII autophosphorylation contributes to, but is not exclusively responsible for, the serotonergic mediation of cocaine preference.

Previous work by Sora and colleagues (1998) using transporter KO mice shows that neither dopamine transport (DAT) nor SERT deletion alone can prevent cocaine CPP (Sora *et al.*, 1998), but that concurrent deletion of both DAT and SERT can stop the development of CPP to cocaine. Past and present data suggest that CaMKII may play a part in the cocaine-induced dopaminergic alterations of the NAcc. This theory is supported by Anderson and colleagues (2008). Increased phosphorylation at the T286 site of CaMKII has been associated with reinstatement of cocaine seeking (Anderson *et al.*, 2008). The T286 site is the same site which has been manipulated in the present study and the mechanism by which phosphorylation is disrupted in Mt mice. This may contribute towards the delay in the establishment of cocaine preference.

Stimulants have been shown to increase extracellular NA levels (Bardo, 1998; White and Kalivas, 1998). Present data showed no difference in the NA response in the NAcc or the PFC in response to cocaine administration in αCaMKII mice, suggesting that there was probably no role for αCaMKII autophosphorylation in the development of cocaine preference.

Cocaine is a stimulant which enhances CNS activity, and is known for its behavioural activating effects (Gass and Olive, 2008; Thomas *et al.*, 2008; Uys and LaLumiere, 2008). Injection of the CaMKII inhibitor KN-93 into the

VTA prior to daily cocaine treatment can impair the development of behavioural sensitisation. The development of sensitisation is attenuated in CaMKII KO mice (Licata *et al.*, 2004). In addition, behavioural hyperactivity is enhanced in αCaMKII KO mice after cocaine (Licata *et al.*, 2004). In the present study, the CPP test design identified a significant conditioned hyperlocomotion in all genotype groups. This suggests that αCaMKII autophosphorylation is not specifically required for this type of cocaine-associated behaviour. Behavioural sensitisation is defined as the increased responsiveness to drugs which develops as a result of their repeated administration (Boudreau *et al.*, 2009). In the case of stimulant drugs, sensitisation to both the locomotor activating effects and incentive-motivational effects can occur (Robinson and Berridge, 2008). The present study revealed similar behaviourally activating effects after an acute cocaine challenge, providing indirect evidence that there are no significant differences in the bioavailability of cocaine between genotype groups. As this behaviour sensitises in all genotype groups, αCaMKII autophosphorylation deficiency does not appear to be important for this behaviour.

NA transmission in the NAcc, VTA and PFC has been reported to be important for psycho-stimulant induced hyperactivity and sensitisation (Nicola and Malenka, 1998; Vanderschuren *et al.*, 1999; Shi *et al.*, 2000). NA is a key transmitter in stimulant-induced arousal (Huether, 1996; Sved *et al.*, 2001) and the observed lack of genotype effect on the acute behavioural activating effects of cocaine may be accounted for by the comparable NA response, as measured by in vivo microdialysis. Since there was no difference observed in the behavioural activating effects of cocaine in the present experiment, it seems unlikely that there would be an adaptation in the noradrenergic system either. Data from the current study showed that NA levels in the NAcc and PFC, conditioned hyperactivity, acute locomotor and sensitisation effects were all stable across groups and over time. Therefore NA appears to be less important for the rewarding and motivational effects of cocaine, but seems to contribute more towards the arousal component and locomotor activating effects of cocaine.

Baseline transmitter levels were measured prior to acute cocaine administration and may provide an explanation for some of the microdialysis results. Basal DA levels in the NAcc of Ht mice were approximately twice as great as either WT or MT levels, and may represent a ceiling effect. Therefore, further enhancement of cocaine-induced DA response may not have been possible in Ht mice and this may help explain the absence of a DA response in the NAcc of Ht mice. Similarly, baseline DA levels were lower in WT compared to Ht and Mt mice in the PFC. The DA response to an injection of cocaine in the Ht and Mt mice were similarly blunted compared to WT mice. WT baseline DA levels were lower which suggests that there may have been a greater capacity for enhanced dopaminergic activity in WT's compared to either Ht or Mt mice. Basal Mt 5-HT levels were approximately twice that of WT mice, and perhaps prevented the further enhancement of activation in both the NAcc and PFC. The same was true of Ht mice in the NAcc, but not the PFC where baseline 5-HT levels were significantly lower than those measured in Mt mice. Baseline 5-HT levels in the PFC cannot offer an explanation as to why Ht mice did not respond to an acute cocaine injection. There was no difference in cocaine-induced NA response in the NAcc and PFC between genotype groups. Baseline NA levels however were not comparable across groups. Together, resting DA, 5-HT and NA levels prior to cocaine treatment may reflect a complex role for αCaMKII autophosphorylation in the resting state of the reward system, influencing response to cocaine administration. CaMKII can phosphorylate a number of intracellular targets including AMPA receptors (Poncer et al., 2002), NMDA receptors (Bayer et al., 2001), L-type $Ca^{2+}$ channels (Dzhura *et al.*, 2000) and tyrosine hydroxylase (Griffith and Schulman, 1988). Tyrosine hydroxylase (TH) is the rate limiting enzyme in DA synthesis, which in turn is a precursor for NA. TH, CaM and CaMKII are co-distributed in the NAcc, and it has been suggested that TH activity and DA synthesis is regulated by $Ca^{2+}$/CaM/CaMKII in the NAcc and other areas (Sutoo *et al.*, 2002). TPH is the initial and rate-limiting enzyme in the biosynthesis of 5-HT and exists in two isoforms, TPH1 and TPH2 (Kuhn *et al.*, 2007). TPH2 is specifically expressed in the CNS and is activated by CaMKII (Hamon *et al.*, 1977; 1978;

Kuhn *et al.*, 1980; Yamauchi and Fujisawa, 1981). Alterations in the activity of CaMKII, and consequently activity of TRP2, could ultimately influence the function of serotonergic nerve endings by increasing or decreasing the amount of transmitter available for release into the extracellular space. Evidence implicates CaMKII in the regulation of DA, 5-HT and NA synthesis in the CNS, suggesting that CaMKII may act as a mediator for basal and cocaine-induced activity for all three transmitters.

Both male and female mice were used in all experiments of the present study, and balanced across groups. Both clinical and rodent studies report sex differences in the behavioural response to cocaine (Festa and Quinones-Jenab, 2004), however the present study did not detect any sex effects. Data were therefore collapsed for the final analysis, although a subtle sex specific role for αCaMKII in the development of cocaine related behaviours cannot be ruled out and is a limitation in the current study. The absence of saline injected groups is a major drawback in the present thesis. Inclusion of saline groups may have helped to identify injection stress-induced increases in behavioural and neurochemical levels, especially since threatening situations and stimuli have been identified as a potential confound (Chapter 2 and Easton *et al.,* 2011). Thus, injection stress cannot be ruled out as a contributing factor and it is not possible to distinguish between responses caused solely by cocaine from those responses caused by the injection procedure (Discussed in full in Chapter 6, 4. Strengths and Limitations).

The glutamatergic system is an important mediator of cocaine reward, increasing synaptic expression of AMPA receptors and thus enhancing neuronal excitability and plasticity. Cocaine increases extracellular glutamate levels (Smith *et al.*, 1995) and is associated with increased sensitivity of dopaminergic neurons to glutamatergic stimulation (White *et al.*, 1995; Zhang *et al.*, 1997) and glutamate subunit expression in the NAcc and VTA (Churchill *et al.*, 1999). Although glutamate was not measured in the present study, alterations in the glutamatergic system may be important and could account for behaviours in the establishment of cocaine preference which cannot be explained by the current data. Further experiments should investigate whether

disruption of αCaMKII autophosphorylation influences the glutamatergic system.

The current study illustrates a complex role of the αCaMKII autophosphorylation mechanism in the development of cocaine preference. αCaMKII autophosphorylation may contribute towards the establishment of cocaine-induced CPP via the monoaminergic system. Present data indicated a difference in the time-course of DA increase in the extracellular space of the NAcc following cocaine administration. This mechanism may facilitate the modulation of CPP. 5-HT is also required for the development of CPP, although the influence on this system via αCaMKII autophosphorylation in the NAcc and PFC is not clear. αCaMKII autophosphorylation did not seem to be required for the development of other cocaine-related behaviours associated with chronic cocaine administration such as conditioned hyperactivity and behavioural sensitisation effects. These effects remained stable over time, as did noradrenergic activity in the NAcc and PFC. There may be other transmitter systems involved in response to cocaine which were not assessed in the present study. Further experiments should investigate the effect of αCaMKII autophosphorylation deficiency on the glutamatergic system in response to cocaine administration. While CaMKII may be important in mediating the effects of cocaine, the specific involvement of αCaMKII autophosphorylation in the development of cocaine preference remains unclear. αCaMKII autophosphorylation cannot solely account for the development of cocaine preference, but may contribute to the fast establishment of some of cocaine's conditioned behavioural effects.

# Ras-GRF2 regulates alcohol consumption by mediating dopamine and serotonin activity in the reward system

## Chapter 5

*Published in part:*

Stacey, D., A. Bilbao, M. Maroteaux, T. Jia, **A. C. Easton**, S. Longueville, C. Nymberg, T. Banaschewski, G. J. Barker, C. Buchel, F. Carvalho, P. J. Conrod, S. Desrivieres, M. Fauth-Buhler, A. Fernandez-Medarde, H. Flor, J. Gallinat, H. Garavan, A. L. Bokde, A. Heinz, B. Ittermann, M. Lathrop, C. Lawrence, E. Loth, A. Lourdusamy, K. F. Mann, J. L. Martinot, F. Nees, M. Palkovits, T. Paus, Z. Pausova, M. Rietschel, B. Ruggeri, E. Santos, M. N. Smolka, O. Staehlin, M. R. Jarvelin, P. Elliott, W. H. Sommer, M. Mameli, C. P. Müller, R. Spanagel, J. A. Girault and G. Schumann (2012). "RASGRF2 regulates alcohol-induced reinforcement by influencing mesolimbic dopamine neuron activity and dopamine release." Proc Natl Acad Sci U S A **109**(51): 21128-21133.

## Abstract:

A novel candidate in the study of addiction was tested to provide further eveidence for the role of this gene in addiction. RasGRF2 belongs to the family of CaM associated guanine nucleotide exchange factors and acts as an ‘on-off’ switch in cells. Ras-GRF2 is involved in the induction of LTD and LTP and Ras-GRF2 KO mice have impaired LTP induction. A recent genome wide analysis of 28,188 people found Ras-GRF2 to be associated with alcohol consumption. Ras-GRF2 KO mice were also found to consume significantly less alcohol and had reduced preference for alcohol compared to their WT counterparts. Data implicate Ras-GRF2 as a novel candidate in the regulation of alcohol-related behaviours. Using in vivo microdialysis, the current study presents evidence for a link between Ras-GRF2 and activity in the monoaminergic system in alcohol consumption. DA and 5-HT alcohol-induced increases were absent in the NAcc and CPu of Ras-GRF2 KO mice. Ras-GRF2 deletion appears to result in the failure of elements of the monoaminergic system to adapt in the normal way in response to alcohol, and may to some extent explain why Ras-GRF2 KO mice consume less alcohol and have a reduced alcohol preference compared to WT controls. Data suggest that Ras-GRF2 deletion may make the animal less vulnerable to alcohol’s rewarding properties and it would be interesting to investigate whether it could potentially serve as a neuro-protective factor against alcoholism.

## 1. Introduction

RasGRF2 belongs to the family of CaM associated guanine nucleotide exchange factors and acts as an 'on-off' switch in cells. Ras-GRF2 becomes active in the presence of CaM, Ras-GRF2 in turn activates the RAS protein which is known to be involved in the MAPK/ERK signal transduction pathway (Agell *et al*., 2002) by allowing the exchange of GDP for GTP, thereby regulating signalling pathways in cells. Ras-GRF2 is known to play a crucial role in glutamatergic signal transduction and the Ras-MAPK signalling cascade. This is activated via NMDA receptors and is involved in the induction of LTD and LTP (Thomas and Huganir, 2004; Li *et al.*, 2006). Ras-GRF2 KO mice are impaired in LTP induction and a recent study has shown that disruption of this pathway alters ethanol drinking behaviour in mice (Stacey *et al*., 2012).

RAS-MAPK/ERK pathway activation typically promotes cell division and is thought to contribute to approximately 30% of all human cancers (Bos, 1989). Crucially, Ras-GRF2 has not previously been associated with any drug of abuse, despite acting on pathways which are also implicated in addiction, such as the dopaminergic system. The RAS-MAPK/ERK pathway has been linked to DRD1 (Tian *et al.*, 2004; Girault *et al.*, 2007), and has also been isolated as a binding partner of DAT (Maiya *et al.*, 2007). These studies suggest a role for Ras-GRF2 at the synapse in the regulation of extracellular transmitter levels and in neurotransmitter release (Bloch-Shilderman *et al.*, 2001). Drugs of abuse 'hijack' the natural reward system in the brain (Nestler, 2002b; Kelley, 2004; Hyman, 2005) resulting in the modification of some major transmitter systems in brain regions including the NAcc and PFC (Pontieri *et al.*, 1995), thereby mediating the rewarding potential of these drugs (Koob *et al.*, 1998b; Wise, 1998; Swanson, 2000; Nestler, 2001; Everitt and Wolf, 2002). Taking this into account, a role for Ras-GRF2 in the development of addictive behaviours certainly warrants further investigation.

Ras-GRF2 has recently been implicated as a novel candidate in the regulation of alcohol consumption (Stacey *et al*., 2012). A genome wide analysis of

28,188 people found polymorphisms of Ras-GRF2 to be significantly associated with alcohol consumption. Ras-GRF2 KO mice also consume significantly less alcohol and have a reduced alcohol preference compared to their WT counterparts and show no difference in taste sensitivity or blood alcohol elimination (Stacey *et* al., 2012). The aim of the current study was to examine the reason for diminished alcohol drinking behaviours seen in Ras-GRF2 KO mice. The central hypothesis states that Ras-GRF2 KO mice do not experience the motivationally rewarding properties of alcohol to the same extent as WT mice, which causes reduced consumption and preference for alcohol. This may be accounted for by dysfunction of neurochemistry in the reward system. This was assessed using in vivo microdialysis measuring alcohol-induced changes in DA, 5-HT and NA levels in the NAcc and CPu, concurrent with locomotor activation. NAcc and CPu were selected for dialysis based on the degree of overlap between the regions in which DA and other monoamines are released in response to alcohol administration, and regions in which Ras-GRF2 is abundantly expressed.

## 2. Materials and Methods

All housing and experimental procedures were performed in accordance with the U.K. Home Office Animals (Experimental Procedures) Act 1986.

### 2.1. Animals

Male wild type (Ras +/+ WT) and null mutant (Ras -/- KO) Rasgrf2$^{tm1Esn}$ (Fernández-Medarde *et al.*, 2002) mice were studied. Mice were generated using a gene targeting strategy (Fig 5.1). The method inactivates the Grf2 locus by targeting its CDC25-H catalytic domain, thereby disrupting the guanine nucleotide exchange factor (GEF) activity of Ras-GRF2. A portion of the CDC25-H domain, a highly conserved region of the gene, was replaced with a PGK-neo cassette via homologous recombination. Absence of gene expression in homozygous mutant animals was confirmed by RT-PCR analysis of brain RNA and absence of protein product was demonstrated by Western blot of brain and lung extracts using an antibody directed against a region of the protein upstream of the disruption. (Fernández-Medarde *et al.*, 2002).

Lambda genomic clones containing several exons of the CDC25-H domain of Grf2 were isolated from a 129 SVJ mouse genomic library (Stratagene). Electroporation of CJ7 embryonic stem (ES) cell line and selection of transfected clones was performed and targeted ES cell clones for the GRF2 gene were injected into C57BL/6 blastocysts. Chimeras were generated in which the mutated allele has been transmitted to the progeny. Mice were kept on a genetic background of 129/S-J and C57BL/6 and bred in a specific-pathogen-free facility.

Animals were individually housed in Tecniplast cages (32cm x 16cm x 14cm), using Litaspen sawdust and nesting materials, (Sizzlenest, Datsand, Manchester UK). Mice were provided with food and water ad libitum, and kept on a 12:12 hour light: dark cycle (lights on at 7.00 am). Experiments were performed during the light cycle between 09:00 and 16:00 h, in a pseudorandom order. Room temperature was maintained between 19℃ and 22℃ at a humidity of 55% (±10%).

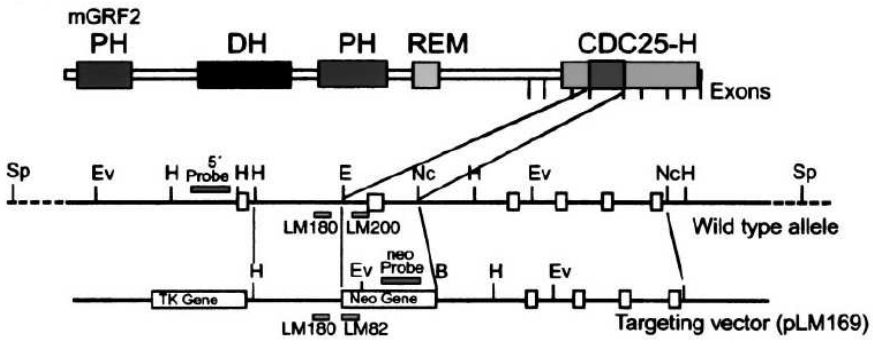



**Figure 5.1:** Schematic representation of the mouse *GRF2* genomic locus and the targeting vector used for replacement of an exon inside the CDC25-H domain. The positions of boundaries of the individual exons coding for the C- terminal portion of Grf2, including the CDC25-H domain, are indicated by the vertical marks. In the schematics for the wild-type allele and the targeting vector, filled boxes represent the exons, whereas open boxes represent the selectable markers (pgk-neo and pkg-tk*)*. Taken from Fernández-Medarde *et al.*, 2002.

### 2.2. Loss of righting reflex (LORR)

Alcohol naïve animals were used for this test (KO: n=5; WT: n=5). For details see Chapter 3, Materials and Methods section 2.6.

### 2.3. In vivo microdialysis

Ras-GRF2 KO mice consume and prefer alcohol less than their WT counterparts (Stacey et al., 2012). The DA system is critical to experience the acute reinforcing effects of alcohol (Koob, 1992; McBride and Li, 1998; Nestler, 2005). Ras-GRF2 has also been linked to DRD1 (Tian *et al.*, 2004; Girault *et al.*, 2007) and isolated as a binding partner of DAT (Maiya *et al.*, 2007). An alcohol challenge produces a change in glutamate levels in the NAcc (Szumlinski *et al*., 2005) and may be associated with altered function of the reward circuitry. However, based on what is known about the reinforcing action of drugs of abuse and the potential involvement of Ras-GRF2, DA

response analysis was chosen over that of glutamate (Spanagel, 2009). In addition, the measurement of extracellular glutamate levels requires a different HPLC set-up with UV detection and cannot be measured alongside the other transmitters of interest. Not having access to an HPLC system with UV detection meant that priority was given to the measurement of extracellular DA, 5-HT and NA. The current study aims to examine whether dysfunction of neurochemistry in the reward system will alter the motivationally rewarding properties of alcohol which might cause Ras-GRF2 KO mice to drink and prefer alcohol less.

*2.3.1. Surgery*

Two guide cannulas (Microbiotech/se AB, Stockholm, Sweden) were aimed at the CPu (A: +0.5; L: ±2.3; V: -2.4 angle ±10º from midline) and the NAcc (A: +1.2; L: ±1.6; V: -4.3 angle ±10º from midline) using coordinates relative to bregma (Franklin and Paxinos, 1997). For details see Chapter 3, Materials and Methods section 2.7.1.

*2.3.2. Procedure*

On the day of the experiment, microdialysis probes of a concentric design (see Chapter 3, Fig. 3.3B), membrane lengths were 2mm for the CPu (MAB 6.14.2.) and 1mm (MAB 6.14.1.) for the NAcc, were inserted into the guide cannulae under a short (3-5min) Isoflurane anaesthesia ($O_2$ at 1L/min, Isoflurane at 3% to induce and 2% to sustain). For details see Chapter 3, Materials and Methods section 2.7.2.

*2.3.3. Acute alcohol effects*

Alcohol naïve male mice were used for this test (Ras -/-: n=8; Ras +/+: n=8). In-vivo microdialysis was carried out at least 5 days after surgery on day one of the experiment during which animals received an alcohol injection (2g/kg, i.p.). This provided an acute neurochemical and behavioural response to alcohol treatment. Once microdialysis experiments were complete, animals were sacrificed by cervical dislocation. Brains were fixed in 4% formaldehyde solution and stored at 4 ℃. Brains were sliced on a microtome and stained

with cresyl violet for analysis of probe placement. Only animals with probe placement within the CPu and the NAcc were considered for data analysis. Since all probes were surgically inserted accurately within the CPu and the NAcc regions, all animals were considered for data analysis.

*2.3.4. HPLC-ED analysis*

All samples were analysed using HPLC-ED to measure DA, 5-HT and NA levels in response to alcohol administration. For details see Chapter 3, Materials and Methods section 2.7.4.

*2.3.5. Behavioural Analysis*

For details see Chapter 3, Materials and Methods section 2.7.5.

**2.4. Statistical Analysis**

All graphical output data is expressed as a mean ± SEM. *Microdialysis:* Baseline neurochemical and behavioural data were analysed using one way ANOVA. Alcohol induced neurochemical effects were expressed as a percentage of the mean of the three baseline samples which were taken as 100% in an attempt to standardise the data across groups. Data were compared using two-way ANOVA with factors genotype (2) and time (13). To compare alcohol effects of certain time points, pre-planned comparisons were performed using Fisher's LSD tests. One way repeated measures ANOVAs and post hoc Fisher LSD tests were performed and used to determine differences between pairs of values within groups. To compare alcohol effects of certain time points pre-planned t-test comparisons, independent by variable, were performed versus a 100% average baseline value. Probability values were subsequently Bonferroni corrected for multiple testing. *LORR:* Data were analysed using one-way ANOVA to compare alcohol effects between groups. The software SPSS 17.0 and Statistica 9 were used. A significance level of $p < 0.05$ was used to test for statistical significance.

As discussed in Chapter 2, Section 2.3. Statistical Analysis, the present study retrospectively used the ‘Resource Equation’ (Mead, 1988) method to quantify the probability that experiments detected biologically important effects. Mead’s resource equation (not using blocking) states:

**E = (total number of experimental units) – (number of treatment combinations)**

**E** was calculated for all experiments in the present study. LORR, **E** = 8; MD, **E** = 14; According to Mead’s rule, **E** should be between 10 and 20. If **E** is less than 10, increasing numbers would lead to good returns. If **E** is over 20, resources may be wasted.

# 3. Results

### 3.1. The hypnotic effects of alcohol are experienced similarly by Ras-GRF2 KO and WT alcohol naïve mice.

The sedating properties of alcohol were experienced in the same way by Ras-GRF2 KO and WT alcohol naïve mice, as a result of an acute alcohol injection ($p>0.05$; Fig. 5.1A). Figure 5.1B illustrates the amount of time mice spent in an ataxic state following a high alcohol dose. This was not different between groups of alcohol naïve Ras-GRF2 KO and WT mice following alcohol administration ($p>0.05$).

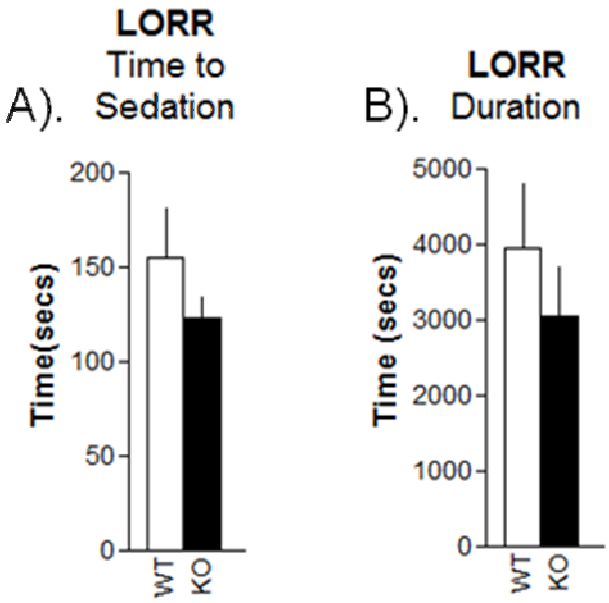



**Figure 5.1:** The hypnotic effects of alcohol are experienced similarly by Ras-GRF2 KO and WT alcohol naïve mice. Loss of righting reflex (LORR) time to sedation **(A)** and LORR duration **(B)** after alcohol (3.5g/Kg, i.p.) treatment.

**3.2. Ras-GRF2 does not alter behavioural responses to alcohol treatment**

Basal locomotor activity was greater in alcohol naïve Ras-GRF2 KO mice compared to WT's ($F_{1,34}$ =7.33, p=0.01; Fig. 5.2A). Behaviour was monitored through-out microdialysis tests, during which an alcohol injection was administered. Two-way ANOVA revealed a significant effect of time ($F_{12,108}$ =2.53, p=0.006) but not of genotype ($F_{1,9}$ = 3.00, p=0.12) or interaction (time x genotype: $F_{12,108}$ = 0.88, p=0.56) on locomotor activity during microdialysis tests (Fig. 5.2B).

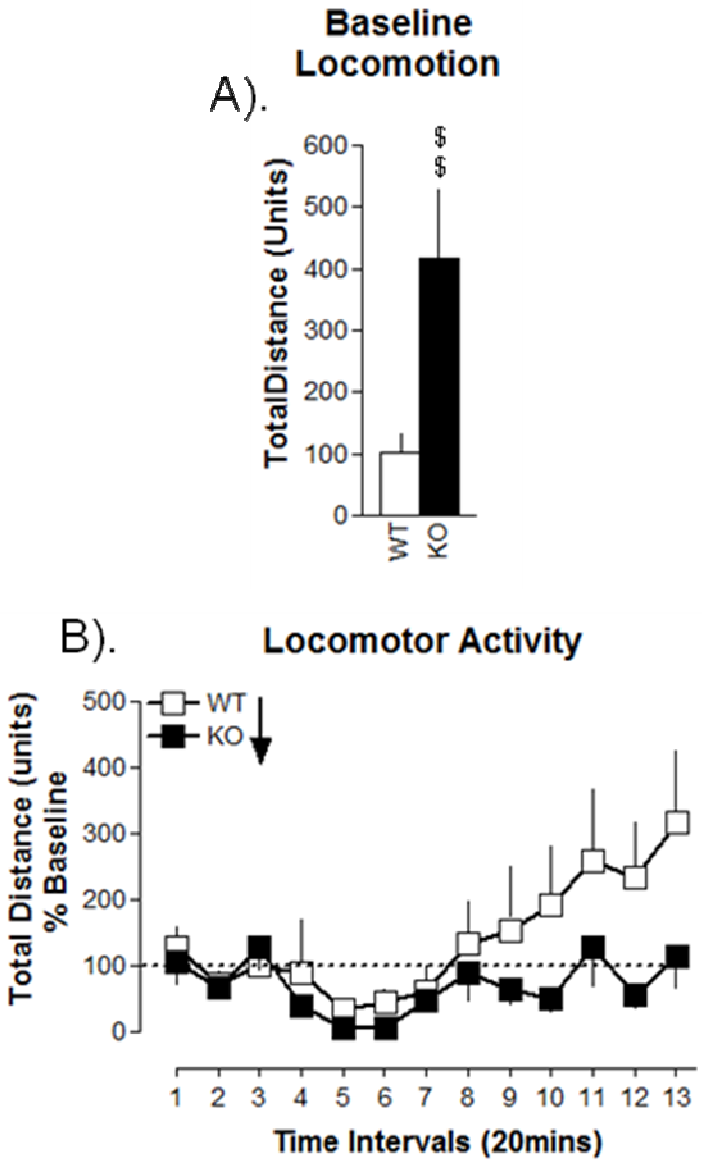


**Figure 5.2:** Ras-GRF2 does not alter behavioural responses to alcohol treatment. Baseline behavioural activity prior to alcohol administration (A). Locomotor activity recorded during microdialysis trial after alcohol treatment (B). Arrow indicates time of alcohol injection. ANOVA; $^{\$\$}p<0.01$ vs. WT.

### 3.3. Elevated basal DA and 5-HT levels of Ras-GRF2 KO compared to WT mice may hinder further alcohol–induced increases in the NAcc.

Basal DA levels in the NAcc of alcohol naïve mice were higher in Ras-GRF2 KO mice compared to WT's ($F_{1,40}$ =20.52, p<0.001; Fig. 5.3A). Baseline DA levels were comparable between groups in the CPu ($F_{1,38}$ =0.06, p=0.80; Fig. 5.3B). Resting 5-HT levels were again greater in the NAcc of Ras-GRF2 KO vs. WT mice ($F_{1,37}$ =23.72, p<0.001; Fig. 5.3C). In contrast, 5-HT levels were lower in the CPu in KO mice ($F_{1,38}$ =14.28, p<0.001; Fig 5.3D). Baseline NA levels were consistently greater in KO mice compared to WT's in both the NAcc ($F_{1,37}$ =18.65, p<0.001; Fig. 5.3E) and the CPu ($F_{1,37}$ =30.88, p<0.001; Fig. 5.3F).

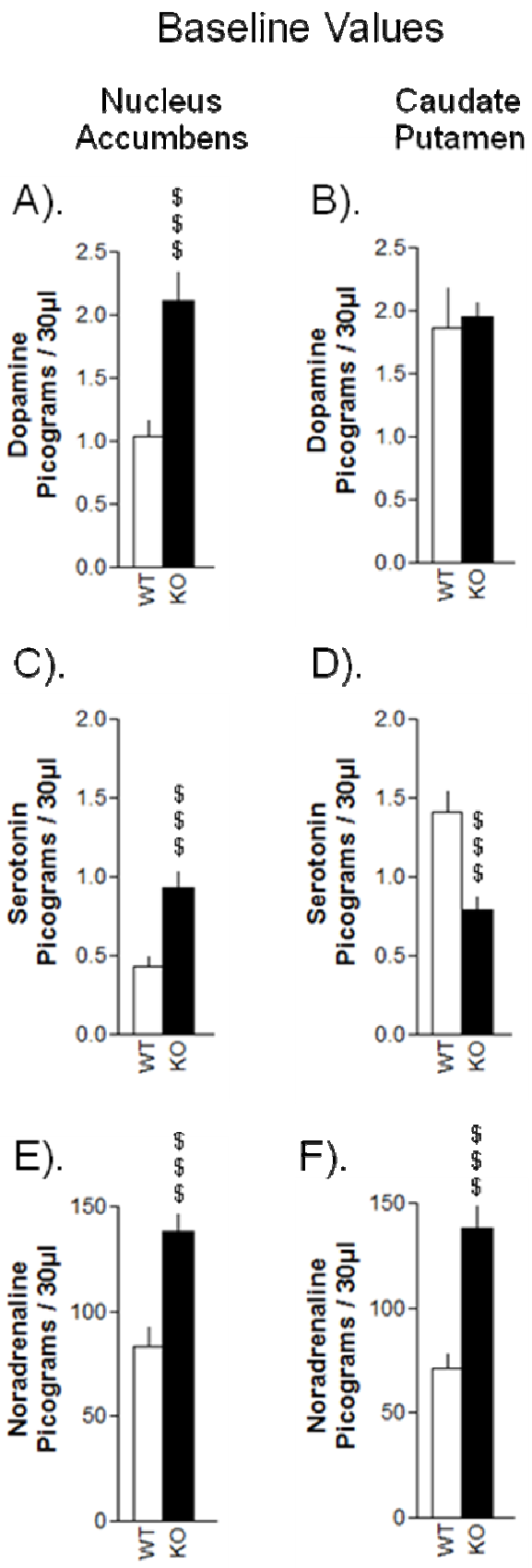

Baseline Values
Nucleus Accumbens
Caudate Putamen
A).
B).
C).
D).
E).
F).
Dopamine Picograms / 30µl
Serotonin Picograms / 30µl
Noradrenaline Picograms / 30µl
WT
KO
$$$$

**Figure 5.3: Baseline monoamine levels.** Bars represent neurotransmitter levels in the NAcc prior to alcohol treatment for DA **(A)**; 5-HT **(C)**; and NA **(E)**. Baseline neurotransmitter levels in the CPu prior to alcohol treatment for DA **(B)**; 5-HT **(D)**; and NA **(F)**. ANOVA; $^{\$\$\$}p<0.001$.

### 3.4. DA response in the NAcc and CPu is absent in Ras-GRF2 KO mice compared to WT animals after alcohol administration

Two-way ANOVA revealed significant effects of time and genotype (time: $F_{12,84}$ =2.86, p=0.002; genotype: $F_{1,7}$ =12.82, p=0.009) and a significant interaction (time x genotype: $F_{12,84}$ =2.80, p=0.003) in the NAcc (Fig. 5.4A). WT DA levels were significantly elevated in response to alcohol treatment for 40 minutes post administration (20 mins: p=0.03; 40 mins: p=0.006) compared to KO levels which remained at basal levels through-out the test.

In the CPu two-way ANOVA showed significant effects of time and genotype (time: $F_{12,84}$ =2.71, p=0.004; genotype: $F_{1,7}$ =6.63, p=0.04) but not significant interaction ((time x genotype: $F_{12,84}$ = 1.37, p=0.20; Fig 5.4B). WT showed a trend for increased DA levels in response to an alcohol injection and was significantly different from KO mice 120 minutes (p=0.04) and 140 minutes (p=0.04) after alcohol was administered (Fig. 5.4B). DA levels in the CPu of Ras-GRF2 KO mice remained at baseline through-out.

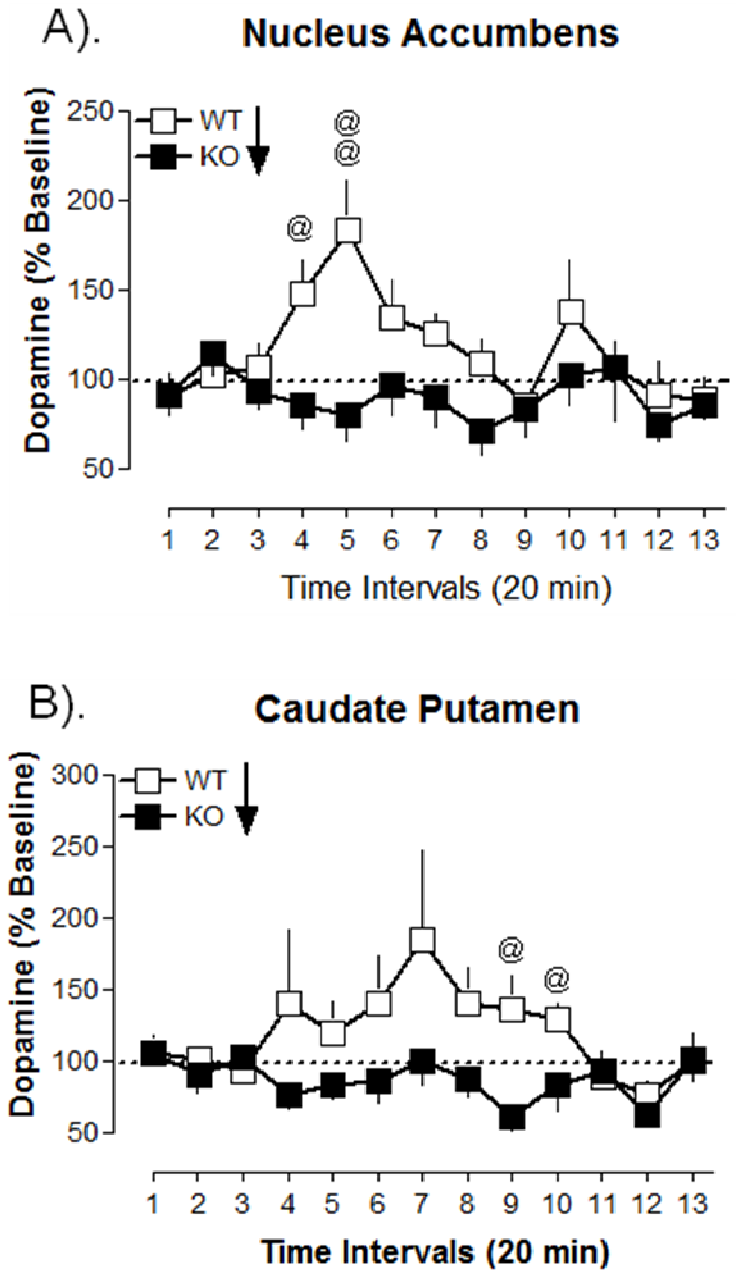



**Figure 5.4:** Dopamine (DA) response in the NAcc (A) and CPu (B) is absent in Ras-GRF2 KO mice compared to WT animals after alcohol administration. Extracellular DA levels as percent baseline in the NAcc and CPu after alcohol treatment. Each point represents a 20 minute time window. Arrow indicates time of alcohol treatment. Planned pairwise comparisons using Fisher's LSD tests between groups at each time point $^{@}p<0.05$; $^{@@}p<0.01$.

### 3.5. There is no 5-HT response to alcohol administration in the NAcc and CPu of Ras-GRF2 KO compared to WT mice

Two-way ANOVA of 5-HT levels in the NAcc revealed significant effects of time and genotype (time: $F_{12,108}$ =2.02, p=0.03; genotype: $F_{1,9}$ =17.41, p=0.002), but no significant interaction (time x genotype: $F_{12,108}$ =1.571, p=0.11; Fig. 5.5A). Immediately after alcohol administration, WT 5-HT response in the NAcc increased compared to Ras-GRF2 KO mice (p=0.03). This trend remained for a further 60 minutes post-alcohol treatment before WT levels returned to baseline. A significant difference in 5-HT response was seen at 160 minutes after alcohol was administered (p=0.02). Ras-GRF2 KO 5-HT levels remained at baseline for the entire test.

5-HT levels in the CPu were also elevated in comparison to KO mice in response to an alcohol injection. Two-way ANOVA showed significant effects of genotype ($F_{1,10}$ =11.81, p=0.006) but not of time ($F_{12,120}$ =0.97, p=0.48), although the time x genotype interaction was significant ($F_{12,120}$ =1.91, p=0.04; Fig 5.5B). WT 5-HT levels were significantly elevated compared to KO mice and increased immediately after alcohol administration. WT and KO genotype groups were significantly different at 20 minutes (p=0.02), 60 minutes (p=0.05) and 120 minutes (p=0.02) post alcohol injection. Just as seen in the NAcc, 5-HT levels in the CPu of Ras-GRF2 KO mice remained at baseline for the entire test.

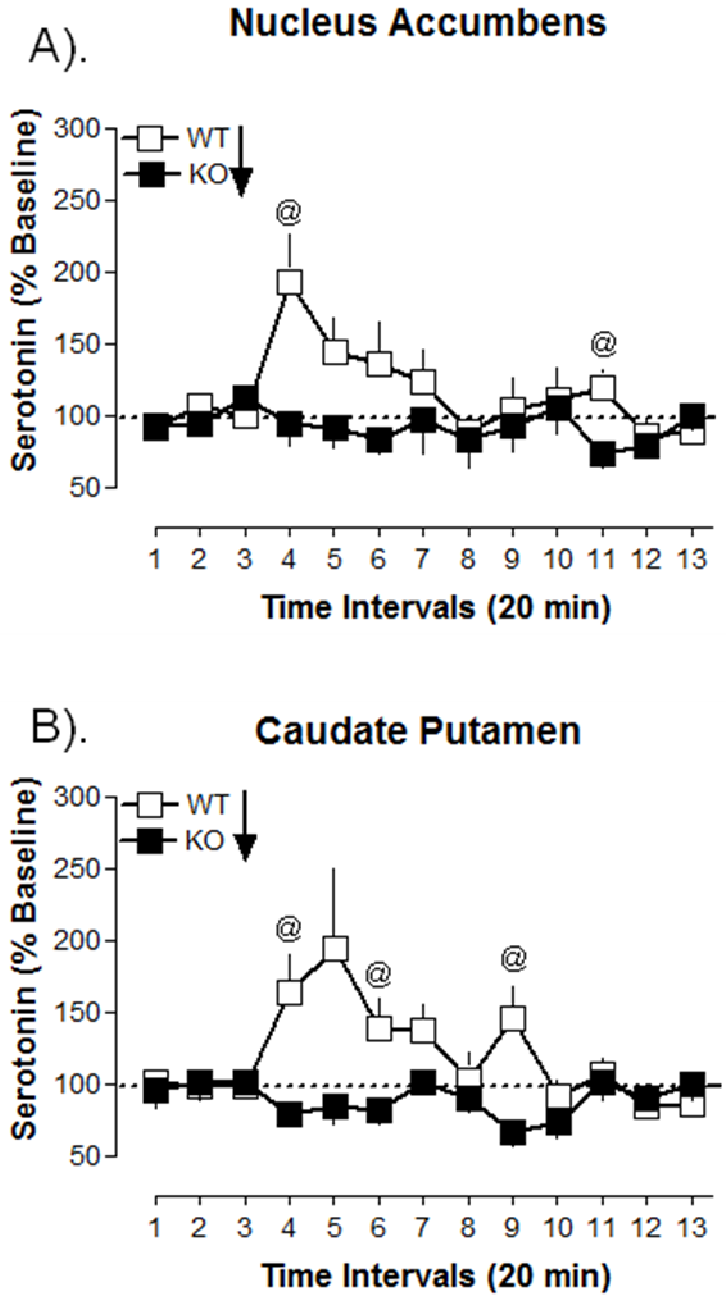



**Figure 5.5:** There is no 5-HT response to alcohol administration in the NAcc (A) and CPu (B) of Ras-GRF2 KO mice compared to WT mice. Extracellular 5-HT levels as percent baseline in the NAcc and CP after alcohol treatment. Each point represents a 20 minute time window. Arrow indicates time of alcohol treatment. Planned pairwise comparisons using Fisher's LSD tests between groups at each time point $^{@}p<0.05$.

### 3.6. Rasgrf2 contributes to noradrenergic activation after acute alcohol treatment.

NA levels in the NAcc increased in all genotype groups in a similar way after alcohol treatment. Two-way ANOVA revealed significant effects of time (time $F_{12,120}$ =7.38, p<0.001; Fig. 5.6A) but not of genotype, or time x genotype interaction. Further exploratory within group analysis using planned pairwise comparisons and suggests that, compared to baseline, WT mice show a significantly increased level of NA at 100 minutes (p=0.03), 120 minutes (p=0.05) and at 200 minutes (p=0.05), after an acute injection of alcohol. KO mice show no significant differences in NA levels after the same alcohol treatment.

NA response to alcohol in the CPu showed a similar profile to those seen in the NAcc. There was a significant effect of time ($F_{12,120}$= 5.03, p<0.001; Fig 1E), but no genotype or genotype x time interaction. Within group analysis showed significantly increased NA levels in WT mice at 100 minutes (p=0.01), 120 minutes (p<0.001), after acute alcohol administration. KO mice do not significantly differ from basal transmitter levels after the same treatment.

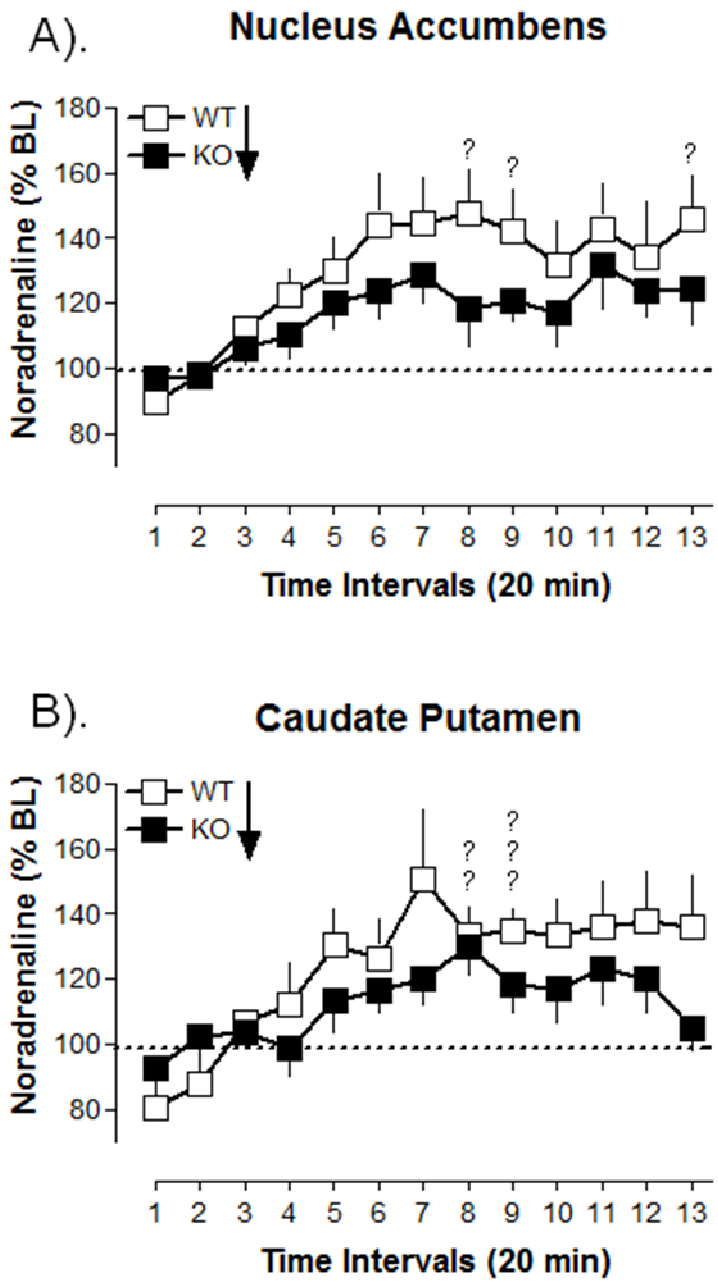



**Figure 5.6:** Rasgrf2 contributes to noradrenergic activation and behavioral activity after acute alcohol treatment. Extracellular NA levels as percent baseline in the NAcc **(A)** and CPu **(B)** after alcohol treatment. Each point represents a 20 minute time window. Arrow indicates time of alcohol treatment. Planned t-test pair-wise comparison vs. baseline of 100%, $^{?}p<0.05$, $^{??}p<0.01$ in WT, $^{???}p<0.001$ in WT.

## 4. Discussion

Ras-GRF2 is a CaM associated guanine nucleotide exchange factor and acts as an ‘on-off switch’ in cells, regulating cell signalling via the MAPK/ERK pathway. Activated through NMDA receptors, the Ras-MAPK signalling cascade is important for the induction of LTP (Thomas and Huganir, 2004; Li *et al.*, 2006), the major cellular mechanism underlying normal learning and memory formation. Disruption of the learning and memory pathway via Ras-GRF2 has recently been associated with alcohol consumption in a genome wide analysis of 28,188 people. In addition Ras-GRF2 KO mice consume significantly less alcohol and have a reduced alcohol preference compared to their WT counterparts (Stacey *et al*., 2012). This work implicates Ras-GRF2 as a novel candidate in the regulation of alcohol consumption. The present study assessed how Ras-GRF2 disruption might contribute towards the previously reported diminished drinking behaviours in Ras-GRF2 KO mice. To expand upon previous data suggesting a role for Ras-GRF2 in the regulation of alcohol consumption and preference, in vivo microdialysis was used to evaluate the motivationally rewarding properties of alcohol by measuring DA, 5-HT and NA levels in the NAcc and CPu, before and after an acute alcohol challenge.

Given the diminished alcohol consumption behaviour seen in both a human population sample and Ras-GRF2 KO mice, it was important to measure the sedating/hypnotic effects of alcohol, as measured by the LORR procedure, to test whether alcohol was being experienced similarly by both KO and WT mice. This behaviour was not affected by Ras-GRF2 deletion and therefore cannot account for the reduced alcohol intake observed in these mice. In addition, locomotor activity was monitored before and after an acute alcohol challenge. Baseline activity levels, monitored prior to alcohol administration, were elevated in the Ras-GRF2 KO compared to WT mice, and could explain, in part, why there was no further enhancement of locomotor activity in KO mice, which remained at basal levels through-out the test.

In order to experience the acute reinforcing effects of alcohol, certain neurotransmitter systems in the reward centres of the brain are recruited

(Koob *et al.*, 1998a; McBride, 2010). In-vivo microdialysis was used to examine monoaminergic responses in the NAcc and CPu to assess which of these transmitters might contribute to the motivational and incentive properties of alcohol. Results revealed that alcohol induced increases in DA and 5-HT levels in both the NAcc and CPu in Ras-GRF2 WT mice may contribute towards the establishment of drinking behaviours previously reported in these mice. The monoaminergic system is consistently implicated in the modulation of alcohol's effects on the reward system (Vengeliene *et al.*, 2008; Spanagel, 2009; Fahlke *et al.*, 2011). DA levels in the NAcc in response to an alcohol challenge, is an indicator of the incentive properties of alcohol (Di Chiara and Imperato, 1988; Koob, 1992). Ras-GRF2 has not previously been associated with any drug of abuse, despite acting on pathways which are also implicated in addiction, such as the dopaminergic system. Ras-GRF2 has been isolated as a binding partner of DAT (Maiya *et al.*, 2007), the primary mechanism for synaptic DA clearance in the striatum and is responsible for the re-uptake of DA into the pre-synapse. The RAS-MAPK/ERK pathway has also been associated with DA D1 receptors (Tian *et al.*, 2004; Girault *et al.*, 2007). These studies suggest an important role for Ras-GRF2 at the synapse in the regulation of reinforcement via dopaminergic signalling (Maiya *et al.*, 2007). Unfortunately these mechanisms cannot explain the difference in DA response seen in the present study since both require initial neurotransmitter release into the synaptic cleft, a response which is absent in Ras-GRF2 KO mice. It may be that Ras-GRF2 is involved in another cellular signalling pathway at the synapse which has not previously been investigated.

The noradrenergic system has been implicated in the long term adaptive changes caused by alcohol consumption (Nutt and Glue, 1988; Fahlke *et al.*, 1999; Berggren *et al.*, 2002), and NA is also involved in the modulation of alcohol's behavioural and neurochemical effects (Fahlke *et al.*, 2011). In the present study, additional exploratory analysis was conducted in order to identify subtle differences which were undetected by statistical analysis methods. WT animals demonstrate a subtle alcohol-induced NA increase in both the NAcc and the CPu compared to basal levels, although there was only a trend for an increase in locomotor activity in WT mice. A

similar increase in NA was seen in Ras-GRF2 KO mice after the same acute alcohol challenge, but, there was no evidence for a change in the locomotor response compared to WT animals. NA is a modulatory transmitter which is involved in the acute and subchronic behavioural effects of alcohol (Fahlke *et al.*, 2011), and may account for the comparable locomotor effects seen in these mice. Ethanol consumption significantly decreases the rate of NA turnover in specific brain areas such as the NAcc, PFC and amygdala (Smith *et al.*, 2008), and such changes are thought to contribute to behavioural changes associated with binge drinking (Smith *et al.*, 2008). NA signaled through the α2 adrenoceptor is involved in the negative feedback control mechanism at neuronal synapses, normally preventing NA release from the presynaptic nerve terminals of the CNS (Schwartz, 1997; Starke, 2001; Brede, 2004). Data may suggest that Ras-GRF2 is capable of controlling the amount of NA present in the synaptic cleft under baseline conditions, although we cannot definitively say in what way this is regulated. Ras-GRF2 activates the Ras protein and thereby stimulates the MAPK/ERK signaling pathway. Adrenoceptors have been shown to differentially alter the activation of the MAPK/ERK pathway (Williams et al., 1998). Williams and colleagues demonstrate that stimulation of the β1 adrenoceptors results in ERK activation, and stimulation of α2 adrenoceptors does not, although this contradicts earlier studies suggesting that α2 adrenoceptors also activate the MAPK/ERK pathway (Howe and Marshall, 1993; Alblas et al., 1993; Winitz et al., 1993).

Baseline transmitter levels in the NAcc and CPu were recorded prior to alcohol administration in Ras-GRF2 KO and WT mice; elevated basal levels have the potential to limit the maximum capacity of alcohol-induced neurotransmitter responses. Basal monoamine levels in the NAcc were consistently elevated in Ras-GRF2 KO compared to WT mice. NAcc DA and 5-HT levels were approximately twice as great as in KO mice, and NA levels were around 50% greater than WT animals. One possibility is that transmitter levels may have reached ceiling levels, limiting further increases in DA and 5-HT following exposure to alcohol. Different basal transmitter levels were observed in the CPu. NA levels remain higher in KO mice than in WT's which

coincided with baseline locomotor activity. DA levels in the CPu were equivalent between WT and KO mice, and 5-HT levels were higher in WT mice. Despite the microdialysis results showing the same pattern across both brain areas, it is possible that basal extracellular monoamine levels may not be as important in the regulation of transmitter response to alcohol in the CPu as they are in the NAcc.

The absence of saline injected groups is a major drawback in the present thesis. Inclusion of saline groups would have helped to identify injection stress-induced increases in behavioural and neurochemical levels. Stress induced changes in catecholamine release are relatively common in the mesolimbic system (Abercrombie *et al*., 1989; Keller *et al*., 1983). However, recent data report contradictory information regarding levels of DA and DA metabolites in the NAcc in response to injection stress (Imperato *et al*., 1993; Johnson and Glick, 1994). Thus, injection stress cannot be ruled out as a contributing factor and it was not possible to distinguish between responses caused solely by alcohol from those responses caused by injection stress. (Discussed in full in Chapter 6, 4. Strengths and Limitations).

Data collected in the present study suggest an important contribution of Ras-GRF2 to DA and 5-HT transmitter release which has not previously been investigated. The current study strengthens the link between this gene and addiction by providing further evidence that disruption of this pathway can alter addictive processes. In this case, Ras-GRF2 does so by modulating activity of the monoaminergic system in response to alcohol administration. Absence of Ras-GRF2 appears to result in the failure of the monoaminergic system to adapt in the normal way in response to alcohol, which may make the animal less vulnerable to alcohol's rewarding properties. One might speculate as to whether Ras-GRF2 deletion can serve as neuro-protective factor against alcoholism.

# Discussion

Chapter 6

## 1. Research Summary

One of the major cellular mechanisms underlying normal learning and memory formation is long-term potentiation (LTP). This process may also be important for drug memories and addiction. At the cellular and molecular level, many drugs of abuse have been documented to induce an LTP-like state in DA neurons of the VTA (Bonci et al., 2003; Saal et al., 2003; Thomas and Malenka, 2003; Borgland et al., 2004; Kauer, 2004), and work by enhancing synaptic transmission, potentiating signals for longer within the cell. In order to investigate the link between these pathways more fully, , the present thesis evaluated whether manipulation of two genes, αCaMKII and Ras-GRF2, can influence measures of drug preference, and the rate at which these preferences are established. This research was carried out using an *in vivo* approach in which specific components of the LTP system were disrupted. Two separate genes (αCaMKII and Ras-GRF2) were studied, each of which code for different CaM activated proteins involved in synaptic plasticity and learning and memory pathways. However, it does not automatically follow that manipulation of these genes and the impact on addiction related behaviours is due to their roles in learning. The thesis initially outlined and aimed to address three central hypotheses:

*Hypothesis 1:*

- *αCaMKII autophosphorylation will alter behavioural responses towards potentially threatening situations.*

The question of differential threat reactivity and anxiety-like responses was raised in relation to αCaMKII autophosphorylation deficient mice. αCaMKII has been implicated in behavioural responses to threat and fearful stimuli (Chen *et al.*, 1994; Hasegawa *et al.*, 2009; Thesis Chapter 2). Prior to commencing experiments, levels of anxiety in response to a novel environment were measured in αCaMKII autophosphorylation deficient mice compared to WT mice. Since the relief of a negative affective state such as anxiety or stress can affect susceptibility to addiction and is closely associated

with compulsive disorders (Koob, 2011), the first step was to further characterise spontaneous behaviours before assessing the mice in other more complex behavioural tasks. The first study was therefore deemed an important control experiment and investigated how αCaMKII autophosphorylation controls behavioural responses towards potentially threatening situations. This is especially important since the internal emotional state has the potential to impact upon behavioural outcomes used in the succeeding studies. By undertaking a comprehensive and systematic battery of spontaneous behaviour tests using homozygous and heterozygous T286A αCaMKII autophosphorylation deficient and WT mice, this molecular mechanism was found to influence locomotor reactivity to novel threatening environments. Therefore, due consideration of this reactivity was made in subsequent studies presented in this thesis.

*Hypothesis 2:*

- *The lack of autophosphorylation should lead to a delay in the establishment of addiction related behaviours.*
- *This should not limit the capacity to establish these behaviours after prolonged drug exposure, or play a role in the maintenance of an addictive state once established.*
- *The initial delay should coincide with changes in the monoaminergic system*

The thesis attempted to address these hypotheses using the same experimental paradigms with two different drugs.

1) Alcohol can increase αCaMKII expression levels in rat cerebral cortex, an effect which can be attributed to $Ca^{2+}$ influx during chronic alcohol exposure (Mahadev *et al.*, 2001). Chapter 3 therefore tested the above hypotheses in relation to alcohol use.

Alcohol drinking was initially diminished in αCaMKII mutants, but adapted and became more in line with the WT response after repeated periods of withdrawal and reinstatement. Data were in-line with the original hypotheses

which stated that αCaMKII autophosphorylation would initially cause a delay in the establishment of addiction related behaviours, but that the mechanism would not play a role in the capacity to establish these behaviours. *In vivo* microdialysis data suggested that αCaMKII autophosphorylation modifies alcohol-induced changes in DA increases in the NAcc of WT mice. Data were again in-line with the original hypothesis which stated that these behavioural alterations would coincide with changes in the monoaminergic system. The blunted DA response seen in Mt mice may in turn be attributed to VTA DA neuron disinhibition. This highlights the involvement of αCaMKII autophosphorylation in the adaptation of the DA system for the establishment of alcohol-related drinking behaviours. However, this cannot be fully determined based on current data. Contrary to what may have been expected given the pattern of establishment of drinking preference, αCaMKII mutants developed alcohol-induced CPP at an accelerated rate, while CPP was established at a classical rate in WT mice (Risinger and Oakes, 1996; Liu *et al.*, 2008).

Taking everything into account, the present study showed that αCaMKII autophosphorylation is important for the establishment of alcoholism-related behaviours. αCaMKII autophosphorylation appears to contribute to both positive and negative reinforcement mechanisms associated with alcohol addiction and this effect may be mediated by a DA-5-HT balance in the meso-corticolimbic system. The positive reinforcing properties of alcohol may be mediated by a VTA driven dopaminergic activation of the NAcc. In αCaMKII mutant mice alcohol preference is reduced and the DA response is absent. However, alcohol CPP is established at an accelerated rate. Deficiency in αCaMKII autophosphorylation may enhance susceptibility to threatening stimuli, an effect which could render animals vulnerable to the negative reinforcing effects of alcohol. This can be linked to serotonergic activation of the PFC. Interestingly, Ht mice showed neither a strong DA nor 5-HT response, suggesting an absence of either positive or negative reinforcement. This is in line with their behaviour since they are the most delayed group in establishing alcohol drinking and alcohol CPP. Taken together, manipulation of αCaMKII autophosphorylation, does appear to alter

the development of alcohol preference and the rate at which preference is established as well as influencing the response of the dopaminergic and serotonergic systems to alcohol.

2) Cocaine reinstatement is associated with CaMKII activation and phosphorylation of CaMKII at the Thr286 site (Anderson *et al*., 2008). Chapter 4 therefore investigates the involvement of cocaine relative to the above hypotheses.

Given the current body of work investigating the role of αCaMKII autophosphorylation in alcohol-related behaviours, it was important to explore whether αCaMKII would play the same role in addictive behaviours when a stimulant such as cocaine is administered, as opposed to a depressant.

Data showed that αCaMKII autophosphorylation deficient (Mt) mice were initially impaired in their ability to develop cocaine-induced CPP compared to Ht and WT mice. Mt mice developed CPP after repeated cocaine treatments and an incubation period during which CPP was consolidated. Data were in-line with the original hypotheses which stated that αCaMKII autophosphorylation deficiency would initially cause a delay in the establishment of addiction related behaviours, but that the mechanism would be less important over time following prolonged drug exposure. While the degree of cocaine preference was altered by the absence of αCaMKII autophosphorylation, other behaviours remained unchanged. Conditioned hyperactivity and the acute locomotor and conditioned behavioural sensitisation effects of cocaine were comparable across genotype groups. Data suggested that establishment of these behaviours may not require the specific involvement of the αCaMKII autophosphorylation mechanism. *In vivo* microdialysis experiments indicated that αCaMKII autophosphorylation deficiency resulted in a difference in the time-course of DA increase in the extracellular space of the NAcc following cocaine administration. This mechanism may facilitate the modulation of CPP and is in-line with the original hypothesis. However, the contribution of the serotonergic and noradrenergic systems in the development of cocaine related behaviours cannot be fully elucidated using current data. There was a difference in resting monoamine

levels in key regions of the reward system between WT, Ht and Mt mice. These data may explain the lack of response of the monoaminergic system and may represent ceiling effects, whereby further enhancement of cocaine-induced response would not have been possible.

The work in this thesis illustrates a complex role of the αCaMKII autophosphorylation mechanism in the development of cocaine preference. Present data suggests that there are other mechanisms at play in the development of addictive states which have not been measured here. Further experiments should investigate the effect of αCaMKII autophosphorylation deficiency on the glutamatergic system in response to cocaine administration. αCaMKII autophosphorylation cannot solely account for the development of cocaine preference, and simply contributes towards the establishment of cocaine-related behaviours.

*Hypothesis 3:*

- *Ras-GRF2 KO mice do not experience the motivationally rewarding properties of alcohol to the same extent as wild-type mice.*
- *This may be accounted for by dysfunction of neurochemistry in the reward system.*

Polymorphisms in the human RASGRF2 gene are significantly associated with alcohol consumption. Ras-GRF2 KO mice also consume and prefer alcohol less than controls (Stacey *et* al., 2012). The studies in Chapter 5 further examined the functional mechanisms responsible for these differences in consumption behaviour.

Data showed that Ras-GRF2 KO mice do not experience the motivationally rewarding properties of alcohol to the same extent as WT mice, which may cause them to drink and prefer alcohol less than WT mice. This may be accounted for by dysfunction of the reward system and is in-line with the original hypotheses. Ras-GRF2 KO mice did not experience alcohol-induced dopaminergic or serotonergic increases in key reward centres of the brain. In addition, noradrenergic alterations in response to alcohol in the NAcc and CPu were attenuated in Ras-GRF2 KO mice. These data suggest an

important contribution of Ras-GRF2 to transmitter release and regulation at the synapse which has not previously been investigated. In this case, Ras-GRF2 does so by modulating activity of the monoaminergic system in response to alcohol administration. Absence of Ras-GRF2 appears to result in the failure of the monoaminergic system to adapt in the normal way in response to alcohol, potentially making the animal less vulnerable to alcohol's rewarding properties. One might speculate as to whether Ras-GRF2 deletion can serve as neuro-protective factor against alcoholism.

## 2. Alcohol vs. Cocaine

The major advantage of assessing the response to two different classes of drugs using the same experimental paradigms in the same genetic model is the ability to examine the broader role of αCaMKII in addiction. While the similarities between alcohol and cocaine are important, it is arguably the differences between the drugs which provide the greatest insight and perhaps the most interesting and fundamental data regarding the specific processes of action of αCaMKII. Alcohol is a rather globally acting drug in the central nervous system compared to cocaine. There are several ethanol targets in the brain (McBride *et al.*, 2002; Harris *et al.*, 2008; Spanagel, 2009) including the NMDA receptors of the glutamatergic system (Lovinger, 1997; Narahashi, 2000), the GABAergic system (Koob *et al.*, 1998a), and DA activity in the mesolimbic system (Di Chiara and Imperato, 1988; Pontieri *et al.*, 1995). Cocaine's effects on the brain are far more localised, it's most specific target being the dopaminergic system (Sofuoglu and Sewell, 2009). Perhaps cocaine's most well documented function is the blockade of monoamine transporters and therefore monoamine (DA, 5-HT and NA) re-uptake (Uhl *et al.*, 2002; Hall *et al.*, 2004; Jones *et al.*, 2009). DA receptors are also heavily implicated in the production of cocaine's psychoactive effects (Montague *et al.*, 2004; Hyman *et al.*, 2006). Taking this into account, it is understandable that in the present thesis there were a number of different responses to some of the paradigms used. The establishment of preference as measured by the CPP test design followed a typical acquisition pattern in response to both alcohol and cocaine in WT mice (Fig 3.9A and Fig 4.1). As initially hypothesised, cocaine-induced CPP was impaired in Mt mice (Fig 4.1). However, alcohol-induced CPP was found to develop at an accelerated rate (Fig 3.9A). The CPP test also revealed that alcohol produced a conditioned sedation effect in Mt's when compared to WT mice, whereas there were no cocaine-induced differences between genotype groups in conditioned hyperactivity. One might conclude that these conditioned effects may contribute towards the manifestation of drug-induced CPP in the present thesis in different, drug specific, ways. As discussed in Chapter 3, alcohol is an anxiolytic drug and may act as a negative reinforcement mechanism. This

is not the case for drugs such as cocaine, and thus would not have the same implications for the establishment of cocaine preference.

Given cocaine's more specific molecular targets in the brain one might anticipate alcohol to affect the monoaminergic system in a more generalised way. Chapter 3 showed that alcohol administration induced changes in dopaminergic, serotonergic and noradrenergic functioning at an acute level, whereas the acute effects of cocaine appeared to be limited to the dopaminergic system (Chapter 4). The response of these systems suggests a differential role for αCaMKII autophosphorylation in the mesolimbic reward system dependent on the type of drug used The vital differences between the central effects of alcohol and cocaine allow for separation of the data in a more straightforward manner. An attempt to fully characterise the underlying action of αCaMKII in the development of addictive behaviours cannot be fully determined using current data and requires further investigation. The data presented in this thesis suggests that αCaMKII autophosphorylation may play a role in the behaviours of specific drugs of abuse as opposed to being universally significant for addictive behaviours. In the current example, it appears as though αCaMKII autophosphorylation may play a greater role in the development of alcohol preference than cocaine preference.

## 3. αCaMKII vs. Ras-GRF2

The thesis aimed to further dissect the relationship between addiction and two separate genes that encode proteins which influence LTP and are involved in synaptic plasticity. Both αCaMKII (Colbran and Brown, 2004; Irvine *et al.*, 2006; Wayman *et al.*, 2008) and Ras-GRF2 have a role to play in the plasticity of the glutamatergic system (Thomas and Huganir, 2004; Tian *et al.*, 2004; Li *et al.*, 2006; Tian and Feig, 2006). They act by influencing LTP, which is thought to underlie normal learning and memory formation as well as being one of the key mediators of alcohol drinking and dependence (Spanagel, 2009). While these genes have different downstream functions, there is some crossover between the gene products and their impact on molecular pathways. When a cell receives an intracellular signal the $Ca^{2+}$ channel becomes active and allows for large $Ca^{2+}$ influx. This results in depolarization of the cell. $Ca^{2+}$ influx through AMPA and NMDA receptors, as well as L-type $Ca^{2+}$ channels, activates $Ca^{2+}$ mediated second messengers such as calmodulin. Following influx, $Ca^{2+}$ binds to calmodulin forming a CaM complex which can in turn bind to and activate both αCaMKII and Ras-GRF2.

Once active, CaMKII is able to directly, or indirectly through the Ras-MAPK pathway, influence a number of intracellular targets including TH, and cAMP response element-binding (CREB) mediated transcription (Xing *et al.*, 1996). The intracellular targets of CaMKII include tyrosine hydroxylase (TH ; Griffith and Schulman, 1988) and tryptophan hydroxylase (TPH). TH is the rate limiting enzyme in DA synthesis, which in turn is a precursor for NA. TH activity and DA synthesis are thought to be regulated by $Ca^{2+}$/CaM/CaMKII in the NAcc and other areas (Sutoo *et al.*, 2002). Tryptophan hydroxylase (TPH) is the rate limiting enzyme in the biosynthesis of 5-HT (Kuhn *et al.*, 2007). The isoform TPH2 is activated by CaMKII phosphorylation and expressed in the CNS (Hamon *et al.*, 1977; 1978; 1981; Kuhn *et al.*, 1980; Kuhn and Lovenberg, 1982; Yamauchi and Fujisawa, 1981; Ehret *et al.*, 1989). Evidence implicates CaMKII in the regulation of DA, 5-HT and NA synthesis in the CNS. This ultimately suggests that CaMKII may act as a mediator for resting levels of transmitter available for release into the extracellular space. Although less

extensively studied, there is also evidence for the involvement of Ras-GRF2 at the synapse. The RAS-MAPK/ERK pathway has been linked to DRD1 (Tian *et al.*, 2004; Girault *et al.*, 2007), and to DAT (Maiya *et al.*, 2007). Previous studies and current data also suggest a role for Ras-GRF2 in the regulation of extracellular transmitter levels and in neurotransmitter release (Bloch-Shilderman *et al.*, 2001). This could have implications for transmitter synthesis, re-uptake and overall transmission.

Further studies should therefore investigate the involvement of αCaMKII and Ras-GRF2 at the synapse. Experiments designed to determine various monoamine receptor and transporter expression levels in the brain may help determine a molecular mechanism of how these genes impact on the reward system. The dopaminergic system may be the initial point of interest since αCaMKII and Ras-GRF2 deficiency have been shown (Fig 3.13A and Fig 5.4) to evoke some interesting alterations in the system in response to alcohol administration.

## 4. Strengths and Limitations

Availability of the mutant mice was a major drawback in the current thesis, it was therefore necessary to use both male and female mice for experiments. Since there were no major sex differences detected in experiments, sexes were combined and the data generated were collapsed for analysis. However, a minor sex specific role for αCaMKII autophosphorylation in threat induced hyperactivity, alcohol and cocaine related behaviours cannot be ruled out. The present study also retrospectively used the 'Resource Equation' method (Mead, 1988) to evaluate whether experiments were sufficiently powered to detect biologically important effects. Using this method it was determined that all experiments in the present thesis had sufficient animal numbers in order to yield good returns and reliable data.

Extracellular levels of glutamate were not measured in microdialysis experiments in the present thesis. Given that the glutamatergic system is one of the main systems affected by αCaMKII and Ras-GRF2, one might have reasoned that glutamate is a transmitter of significant interest and importance to the current study. Glutamate levels in the NAcc are altered upon acute alcohol administration and can affect the function of the reward system (Szumlinski *et al*., 2005). The activation of AMPA receptors in the NAcc reinstates cocaine seeking (Kalivas *et al.*, 2005; Schmidt *et al.*, 2005), and conversely antagonism of AMPA receptors attenuates cocaine reinstatement (Cornish *et al.*, 1999; Park *et al.*, 2002; Suto *et al.*, 2004). Data heavily implicates glutamatergic influence in drug addiction. The reinforcing actions of drugs of abuse are dependent upon the activity of mesolimbic DA neurons in the VTA and their projections into the ventral striatum and PFC (Spanagel and Weiss, 1999). The DA system is described as being of crucial impotance in order to experience the acute reinforcing effects of alcohol (Koob, 1992; McBride and Li, 1998; Nestler, 2005), with alcohol typically inducing an approximate 20% increase in DA levels in the NAcc (Doyon *et al.*, 2003). Calcium/calmodulin dependent protein kinases and the Ras/mitogen-activated protein kinases are also involved in DA signalling, especially in relation to stimulant drugs (Licata and Pierce, 2003; Licata *et al*., 2004). Based on what

is known about the reinforcing action of drugs of abuse (Spanagel, 2009), measurement of the DA response was chosen over that of glutamate. In addition, an HPLC system with UV detection was not available and therefore glutamate was not assessed in this thesis but the importance of such studies is acknowledged.

Following microdialysis experiments, Stacey and colleagues (Stacey *et al*., 2012) investigated whether altered synaptic glutamate input into the VTA could explain altered extracellular DA levels. The authors recorded excitatory postsynaptic currents of mesolimbic DA neurons by activation of AMPA and NMDA receptors in the presence of bicuculline, a $GABA_A$ receptor blocker. They reported no significant difference in AMPA:NMDA ratio between Ras-GRF2 KO and WT controls. Stacey and colleagues (2012) also recorded DA neurons in current-clamp mode and measured action potentials elicited by increasing depolarizing current injections. They reported a reduced excitability of DA neurons of Ras-GRF2 KO mice compared to WT animals. Data indicate that impaired regulation of DA neuron excitability may provide an explanation for the deficient release of DA in the NAcc in the current thesis.

The absence of saline injected groups for each genotype in all microdialysis experiments is a major limitation in the present thesis. Inclusion of saline groups would have determined whether injection stress alone induced behavioural and/or neurochemical changes, especially since threatening situations and stimuli have been identified as a potential confound (Chapter 2 and Easton *et al.,* 2011). Thus, injection stress cannot be ruled out as a contributing factor, and the specific responses caused by either alcohol or cocaine cannot be fully determined. Stress induced changes in catecholamine release are relatively common in the mesolimbic system (Abercrombie *et al*., 1989; Keller *et al*., 1983). However, recent data revealed no consistent alterations in the NAcc in basal DOPAC and HVA levels between naive rats, and rats that had been injected once a day for four days, or twice a day for four days (Johnson and Glick, 1994). Findings suggest that handling and/or saline injections twice a day increases basal DA metabolism in the striatum, but not in the NAcc (Johnson and Glick, 1994). Imperato and colleagues also

show no significant differences in basal output of DA and the DA metabolites DOPAC and HVA in the NAcc on days 1 and 6 of testing between control rats and rats which had been stressed for the 5 days in between samplings (Imperato *et al*., 1993). Taking all this information into account, as well as carefully considering the high cost and limited availability of mice, the decision was made to replicate the more recent study design adopted by the Spanagel (Spanagel et al., 2005b) and Szumlinski research teams (Szumlinski et al., 2005). This approach subsequently appears to be flawed in its design, especially when one considers the underlying behavioural phenotype of the αCaMKII mice described earlier (Easton *et al*., 2011; Chapter 2).

The use of homozygous and heterozygous αCaMKII autophosphorylation deficient and WT mice is a major strength of the current thesis, and provides a very thorough investigation of the specific role of this mechanism in addiction related behaviours. The inclusion of the heterozygous mice has proven to be very informative in the present thesis, especially when one considers that a knock-out or knock-down model can have its own drawbacks. Knock-out mice may implement mechanisms to compensate for the absence of the gene/protein in question, and may not therefore be an accurate representation of true variation in gene function. This is where the use of heterozygous animals can be helpful in determining a more precise portrayal of the deficiency of certain genes. Heterozygous mice are sometimes expected to produce a response somewhere between that of the wild-type and knock-out mice. This is known as an ‘additive’ effect of the given gene/protein being investigated, i.e. the more copies one has of a specific gene, the greater the response. However, this is not always the case, and heterozygous mice can exhibit characteristics in other ways. A response may be considered as either ‘dominant’ or ‘recessive’ if the heterozygous mouse displays a response similar to that of either the wild-type or the knock-out mouse. Heterozygous mice may also develop a response termed ‘hybrid vigour’ or ‘heterosis’ and describes the superiority of heterozygous genotypes in certain characteristics when compared to the corresponding parental homozygotes. Inclusion of heterozygous mice in the current thesis makes interpretation of the data more

complex. However, it was possible to acquire a more detailed awareness of αCaMKII in particular and its role in addiction-related behaviours, especially when attempting to disentangle the positive and negative reinforcing effects of alcohol in Chapter 3.

The development of gene targeting strategies means that a mutant organism can be created that completely lacks the gene product. However, this method can also lead to a range of secondary phenotypic changes e.g. developmental, physiological or even behavioural (Büeler *et al.,* 1992; Gerlai, 1996; Wolfer *et al*., 2002), termed a 'congenic footprint' effect. These differences may not have anything to do with the gene of interest and may be a result of the organisms' genetic background. Most gene targeting is carried out in embryonic stem cells from the 129 mouse strain (Silva *et al*., 1997). Mice will therefore have a set of chromosomes from the 129 strain and the C57BL/6 strain, as in the transgenic mouse models used in the present thesis. The probability of recombination is inversely related to the distance between loci, thus the 129-type alleles, which are closest to the mutant gene, will remain together with the mutant gene of interest (Gerlai, 1996). This implies that if the mutation is detected in a mouse, then there is a high probability that the mouse will also carry the 129-type flanking genes (Gerlai, 1996; Wolfer *et al*., 2002). This is a very important consideration when choosing to work with transgenic mouse models and a key issue when discussing the effects of gene products. A solution to this problem is to backcross the mice to create a congenic strain that carries both the mutation and the desired genetic background (Gerlai, 1996). In the present thesis both αCaMKII and Ras-GRF2 mutant mice were kept on a mixed background, this was largely due to reasons of fecundity. This may potentially explain the inconsistency seen in the behaviour between mice and the amount of variation sometimes seen within specific genotype groups. For example, Figure 4.7 showed 5-HT response in the NAcc and PFC in αCaMKII autophosphorylation deficient mice after cocaine administration. WT data was considerably more variable than that of both Ht and Mt mice and may be due to the mixed genetic background.

Data in the present thesis combines an extensive methodological array of experiments, the use of elaborate behavioural and neurochemical protocols provide evidence for a role of αCaMKII and Ras-GRF2 in addiction pathways. An important role of αCaMKII autophosphorylation in the development of alcohol and cocaine related behaviours, and for the novel role of Ras-GRF2 in the response to alcohol has been suggested by the work in this thesis. Of particular note here, and of considerable interest to the addiction field, is firstly the role of αCaMKII in mediating the differential reinforcing effects of alcohol, predominantly the implications of αCaMKII in the negatively reinforcing mechanistic effects of alcohol use. Secondly, the proposal of Ras-GRF2 as a novel candidate in the mediation of alcoholism, a mechanism which appears to act by modulating monoaminergic system activation in important brain regions associated with reward.

# 5. Future directions

## 5.1. αCaMKII autophosphorylation

While the present thesis revealed several important and novel functions of αCaMKII autophosphorylation in spontaneous and addictive behaviours, there are many questions which remain unclear, thereby warranting further investigation. While the alcohol study has been developed across a series of experiments, research into the role of αCaMKII autophosphorylation on the behavioural effects of cocaine use remains relatively underrepresented. Following up on the current thesis, there are plans in place to repeat c-Fos analysis of the VTA using another drug, cocaine. It would also be invaluable to look at cocaine self-administration, withdrawal and reinstatement/relapse behaviours in αCaMKII mice. Expanding the current battery of tests to more closely mimic the alcohol study design as far as possible, would allow for a more accurate assessment of the drug specific role of αCaMKII autophosphorylation in addiction. Once the functions of this integral molecule have been more precisely elucidated, it would be of considerable benefit to support this vast amount of evidence in an animal model with an exploration of an appropriate human sample to see if the conclusions that have been drawn based on current findings are clinically valid.

## 5.2. Ras-GRF2

Investigation into the role of Ras-GRF2 in alcohol consumption is relatively unexplored territory. It is therefore difficult to speculate concerning the precise involvement of Ras-GRF2 at this early stage. There is evidence for involvement of Ras-GRF2 at the synapse since the RAS-MAPK/ERK pathway has been linked to both DRD1 (Tian *et al.*, 2004; Girault *et al.*, 2007), and to DAT (Maiya *et al.*, 2007). Previous studies and current data also suggest a role for Ras-GRF2 in the regulation of extracellular transmitter levels and in neurotransmitter release (Bloch-Shilderman *et al.*, 2001), but could also have implications for synthesis, re-uptake and overall transmission. Ras-GRF2 has been implicated pre- and post-synaptically and further studies should involve

the inclusion of DA receptor and DAT expression levels in the brain In order to determine a molecular mechanism of how Ras-GRF2 might affect basal and induced DA activity in the NAcc.

### 5.3. Other approaches

The current thesis identifies two targets worthy of further in-depth investigation. There are several experimental approaches which may be utilised in order to look at the degree of involvement of these and other genes in the establishment of addictive behaviours. It may be of considerable interest to the addiction field to repeat and thereby extend current studies by using other drugs of abuse e.g. opiates or nicotine, and by performing microdialysis in additional brain areas of interest e.g. the VTA. Data would provide a more thorough and specific characterisation of the involvement of these genes in reward pathways and thus the establishment of addictive behaviours. Targets may also be used to develop specific strategies for the rescue of addiction phenotypes. For example, highly sophisticated techniques such as intra-cerebral injections using adeno-virus' or antibodies may be used to alter protein and/or gene expression levels (Fernandes *et al.*, 2012). Such specialised procedures, along with pharmacological intervention strategies may help in identifying the precise mechanisms involved in the development of addiction and determine whether the rescue and subsequent treatment of addiction is possible through these targets. Although αCaMKII and RasGRF2 have been shown to play roles in learning and memory, unfortunately current data cannot directly support that these alterations in gene function have any consequences on the addiction related behaviours described here. In order to assess this further, experiments looking explicitly at LTP and learning mechanisms in these transgenic mouse models would need to be performed.

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